PHD

Lipid Drug Delivery Systems and Their Fate after Oral Administration

Al Sukhun, Rajaa Abed El-Kader

Award date:
2002

Awarding institution:
University of Bath

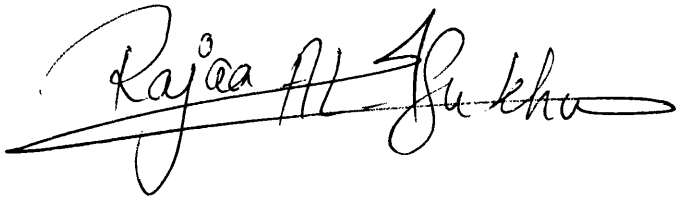
Lipid Drug Delivery Systems and Their Fate after Oral Administration

Submitted by *Rajaa Abed El-Kader Al-Sukhun*

for the degree of PhD of the University of Bath

2002

COPYRIGHT



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Dedication

For my parents and my sister..... .

Acknowledgement

My sincere thanks go to my supervisor Prof. Colin Pouton for his guidance throughout my time at Bath. I have thoroughly enjoyed the work and this is in no small part due to Colin's relaxed approach and his constant encouragement.

I am grateful to Dr. Steven Moss for his encouragement and advice.

I would like to thank many other members of the Department of Pharmacy and Pharmacology, University of Bath, especially Dr. Mike Threadgill for his scientific advice once he read the thesis and his keenness to finish my thesis on time, Dr. Anthony Smith for his offer of help in correcting the thesis grammar and punctuation and Dr Michael Arnott for his help to read some chapters of thesis.

I owe Prof. Abdalla El-Khawad thanks because he is the one who helped me hand-on-hand with my parents to start my Ph.D and enabled me to do some experiments at Al-Razi Pharmaceutical Company and/or Petra University in Amman / Jordan.

I am indebted to Mrs Sue Johnson and Mr Micheal Mason for their friendship, sense of humour and support, whenever called upon.

I also owe thanks to the technical staff of the pharmacy department especially Mr Kevin Smith for his expertise using HPLC and in printing posters, Mr Don Perry and Mr Rod Murray for helping to sort out those practical problems which can otherwise ruin hours of work.

Nothing can describe my sincere gratitude and thanks to my parents for their financial and emotional support throughout my Ph.D. Also, thanks to my brothers, Dr. Jehad Al-Sukhun and Eng. Raid Al-Sukhun, for being supportive and helpful.

I cannot thank enough my sister, sincere friend, Dr. Sana Al-Sukhun for her lovely, encouraging, and enthusiastic words. Having her next to me played a major part and made my life a lot easier throughout my research.

Grateful thanks to Dr. David Perkin and the Esther Parkin Trust for their financial support for the last two years.

Finally, thanks to all my friends either in Amman or in Bath for putting up with it all!

Abstract

A novel class of lipid formulation was investigated comprising GRAS (generally regarded as safe) materials. The formulations were all 'surfactant-free' (S-F) formulations, and also referred to as 'Type IV' lipid formulations. These formulations were isotropic, transparent, thermodynamically stable at room temperature and typically composed of > 50 % of mixed mono-, di- and tri-glycerides, > 30 % medium chain fatty acids oil and < 20 % hydrophilic co-solvent.

At equilibrium, S-F formulations enhanced the solvent capacity of corticosteroids (log P > 3) and hydroxy benzoate derivatives over type II SEDDS and type III SEDDS, but generally were not superior solvents to mixtures of mono-, di- and tri-glycerides (Imwitor 988® and or Capmul MCM®) alone, for lipophilic steroids (log P < 3). In general, type III SEDDS which were composed of high hydrophilic content (hydrophilic surfactant, HLB > 12, and hydrophilic co-solvent), were also better solvents for most steroidal compounds and hydroxy benzoate derivatives than type II SEDDS and type I SEDDS formulations.

Surfactant with HLB > 12 inhibited lipolysis of MCT and mixed glycerides when the concentration of surfactant exceeded 40 % w/w. Hydrophobic surfactants (HLB <10) did not inhibit lipolysis. Thus, the digestibility of dispersions formed by self-emulsifying systems would be dependent on the surfactants used and the quantity of TG available for lipolysis. Co-solvents did not appear to influence lipolysis, once the formulations had dispersed.

Phase separation of lipid formulations following their dispersion in simulated intestinal fluid was studied. The lipid formulation behaviour was dependent on monoglyceride content. When sufficient monoglyceride (> 60 %w/v) was present demulsification and phase separation was noticed and was found to be dependent on the presence of phospholipid. This resulted in sedimentation of the phase rich

monoglyceride and water. The presence of triglyceride stabilised the formation of mixed micelles, which remained in a finely dispersed state. This unexpected phase separation is likely to have a considerable effect on the fate of drug dissolved in SEDDS formulations. The high concentrations of monoglyceride may be disadvantageous and could possibly result in precipitation of drug.

Contents

List of contents

List of Tables

List of Figures

Abbreviations

ACE	Acetonitrile
App.	Appendix
AUC	Area under the curve
BBM	Brush border membrane
BS	Bile salt
C.	Corn oil
CE	Cholesteryl ester
Chap.	Chapter
CM	Chylomicrons
CMC	Critical micellar concentration
CRH 40	Cremophor RH 40
Cryo-tem	Cryo–transmission electron microscopic.
DG	Diglyceride
DGAT	Diglyceride acyl transferase
EP	European pharmacopea
FA	Fatty acid
FABPm	Fatty acids binding protein
FaSSIF	Fasted state simulated intestinal fluid
FeSSIF	Fed state simulated intestinal fluid
FCO	Fractionated coconut oil
g	Gram
form.	Formulation
GI	Gastrointestinal.
GIT	Gastrointestinal tract
Gly.	Glycerine
GRAS	Generally regarded as safe
GPR	General purpose reagent
H.A.	Hydrocortisone acetate
hGL	Human gastric lipase
HLB	Hydrophilic – lipophilic balance
hPL	Human pancreatic lipase
Hydr(H)	Hydrocortisone
I 988	Imwitor 988
INC	Incorporated
IS	Internal standard
IVIV	In vitro – In – vivo
KcL	Potassium chloride
L	Pancreatic lipase
LC	Lecithin
LCT	Long chain triglycerides
M 812	Miglyol 812
MCT	Medium chain triglycerides

MG	Monoglyceride
MGAT	Monoglyceride acyl transferase
min	Minutes
MWT	Molecular weight
μM	Micrometer
NaoH	Sodium hydroxide
NaTDC	Sodium taurodeoxycholic acid
nm	nanometer
O/W	Oil / Water
1 M	One molar
PEG 400	Polyethylene glycol 400
PG	Propylene glycol
Phs. buffer	Phosphate buffer
PL	Phospholipid
Prog.	Progesterone
QLS	Quasielastic light scattering
RT	Retention time
S	Substrate
S_o	The aqueous solubility either in tris – maleate buffer or in water
SCT	Short chain triglycerides
SEDDS	Self – emulsifying drug delivery system
SEDDSs	Self – emulsifying drug delivery systems
S-F	Surfactant – free
S_i	Intrinsic solubility
T 80	Tween 80
T.A.	Testosterone acetate
Tab.	Table
TDC	Taurodeoxycholic acid
Tes.(T)	Testosterone
TG	Triglycerides
TGAT	Triglyceride acyl transferase
Trans. P	Transcutol P
TTO	Tagat TO
UV	Ultraviolet
UWL	Unstirred water layer
VLDL	Very low density lipoprotein
W/O	Water / Oil
W.out Cos.	Without co-solvent

Origins

In recent years much attention has been focused on lipid formulations, with particular emphasis on self-emulsifying drug delivery systems (SEDDS) to improve oral bioavailability of lipophilic drugs (Constantinides, 1995; Pouton, 1997). Poor oral bioavailability of lipophilic drugs may be due to a combination of factors including; poor solubility / dissolution, degradation in the gut lumen, poor membrane permeability or presystemic elimination (O'Driscoll, 1996). The balance between considerations pertinent to dissolution and membrane permeability has led to the development of recent biopharmaceutical drug classification system proposed by Amidon *et al*, (1995) where by drugs are considered to belong to one of four categories depending on their dissolution and membrane permeability characteristics. There is now general agreement that lipid formulations can enhance the oral bioavailability of a range of class II or class IV drugs, particularly those with a solubility of less than 100 μg / ml (Craig *et al*, 2000).

From a practical and aesthetic standpoint, lipid based formulation are ideally prepared as a unit dose form which could be filled into either a sealed hard or soft gelatin capsule (Pouton, 1997). Consequently, much attention has focused on the use of SEDDS, which can be prepared as isotropic mixtures of oil, surfactant, co-surfactant and drug. They form fine oil-in-water emulsions when introduced into aqueous media under mild agitation. The digestive motility of the stomach and intestine provide the agitation necessary for self-emulsification *in vivo* (Shah *et al*, 1994). Factors controlling the *in vivo* performance of SEDDS include digestibility, particle size of the droplet, and the influence of polarity of the oil droplets on drug release into the aqueous phase. Through the normal process of fat digestion (lipolysis), mixed bile salt and lecithin micelles are secreted from the gall bladder and solubilize the breakdown products arising from lipolysis, such as monoglycerides and fatty acids. Small oil droplets provide a large interfacial area for pancreatic lipase to hydrolyse triglycerides and the mixed micelles formed can act as a reservoir of breakdown products by maintaining them in solution in the lumen. When fatty acids

or monoglycerides are absorbed from the aqueous contents of the gut, dissolved material can be more freely made available by partitioning from the mixed micelle (Solomon *et al*, 1997).

The maximum oral bioavailability of a drug from a lipid-based dose form will depend on the composition of the formulation and is likely to involve the following processes: (i) digestion of the medium or long-chain triglyceride lipid, (ii) drug solubilization either within the bile salt micellar milieu of the pre- or post-prandial intestinal tract, or other formulation-derived colloidal structures, (iii) dissociation of solubilized drug from either bile salt or formulation-derived micelles to support absorption, and (iiii) a potential effect of either formulation excipients, or drug, on enterocyte-based metabolism and/or efflux pumps (Charman, 2000).

The ability of lipids and/or food to enhance the bioavailability of poorly water-soluble drugs has been well reviewed recently by Porter and Charman (1997, 2001). In concert with the recent increase in lipidic formulations, there has been an increase in the number of attentive mechanisms proposed by which absorption of lipophilic drugs can be enhanced, namely:

- Increase in effective lumenal drug solubility (Porter and Charman, 2001).
- Improved drug dissolution by reducing the gastrointestinal transit, thereby slowing delivery of the lipophilic drug to the absorptive site (Constantinides *et al*, 1996).
- Increased intestinal epithelial permeability (*i.e.* changes to the physical barrier function of the GI) due to the presence of various combinations of lipids, lipid digestion products, and surfactants (Swenson and Curartolo, 1992).
- Increased tight junction permeability and decreased/inhibited p-glycoprotein drug efflux (*i.e.* changes to biochemical barrier function)

(Nerurkar *et al*, 1996; Yu *et al*, 1999).

- Enhanced intestinal lymphatic transport resulting in a reduction in first pass metabolism of highly lipophilic drugs (Porter *et al*, 1997, 2001).

The conversion of this scientific potential into commercial products, however, has only recently been developed from simple lipid solution formulations, such as those commonly used to deliver fat soluble vitamins, towards effective self - emulsifying or self - microemulsifying lipid formulations for cyclosporin (Neoral®), and subsequently ritonavir (Novir®) and saquinavir (Fortovase®).

Though the type of lipid formulation and the properties of the excipients used will have a critical influence on the fate of the drug in the gut, there is insufficient data in the literature to allow formulators to make appropriate choices. The aim of lipid formulations is to ensure that the drug remains in solution throughout gastrointestinal transit.

More information is needed on:

- The solvent capacity of formulations and how each excipient influence solvent capacity.
- The influence of excipients on the changes in solvent capacity after dispersion of the formulation.
- The interaction between dispersed formulation and the digestive system, and the fate of drugs after digestion of oily components.

Introduction

1.1 Introduction to lipid formulations

In recent years there has been a considerable increase in the number of drugs that are pharmacologically active but poorly absorbed from the intestine, and it would be valuable to find ways of overcoming the in poor bioavailability (Charman *et al*, 1997). The Biopharmaceutics Classification System (BCS) (Amidon *et al*, 1995) categorized drugs into four classes as shown in Table 1.1, depending on their solubility and permeability characteristics, which are the underlying parameters controlling drug absorption. According to this classification, Class I (*High Solubility-High Permeability*) drugs should be more than 90 % absorbed and the rate-limiting step to drug absorption is the drug dissolution and/or gastric emptying *e.g.* paracetamol, where the dissolution step is rapid. Class II (*Low Solubility – High Permeability*) drugs are those with low solubilities but have the potential to be completely absorbed due to high low membrane permeability. Examples include steroids and cyclosporin A. Class III (*High Solubility – Low Permeability*) drugs are the mirror image of Class II drugs (Dressman *et al*, 2000). They have good solubility but low permeability through the gut wall makes the absorption incomplete. Examples include α - methyldopa and atenolol. Both the rate and the extent of absorption of class II drugs may be highly variable; this variation will usually be due to the variables of gastrointestinal transit, luminal contents, and membrane permeability rather than dosage form factors. Class IV (*Low Solubility -Low Permeability*) drugs have poor solubility, permeability and often have poor bioavailability like (furosemide, griseofulvin and hydrochlorothiazide) (Löbenberg *et al*, 2000). This classification scheme provides a basis for establishing *in vitro-in vivo* correlations and for estimating the absorption of drugs based on the fundamental dissolution and permeability properties of physiologic importance. It has been reported that oral bioavailability of poorly-water soluble drugs (Class II) may be improved due to the presence of lipid after a high fat meal (Crounse, 1961, Crounse, 1963) or by reformulating in oily vehicles such as digestible triglycerides or a

mixture of oils and surfactant (MacGregor *et al*, 1997).

Drug dissolution is a prerequisite to drug absorption for almost all drugs given orally (Amidon *et al*, 1995). The hydrophobic drug is presented in a crystalline state within the dosage form leading to a slow dissolution within the lumen of the gastrointestinal tract and perhaps insufficient time to allow complete absorption. Formulation of hydrophobic drugs in a lipid-based product can avoid the dissolution step which leads to improved bioavailability or gives rise to a more consistent profile of drug absorption with time. With all these formulations, the precise mechanism of enhancement of bioavailability is poorly understood. Various physiological mechanisms have been proposed to explain the beneficial effect of co-ingested lipid on the absorption of water insoluble compounds including: altered gastrointestinal motility, increased bile flow and drug solubilization (Bates and Sequeria, 1975), increased mucosal permeability (Muranushi *et al*, 1980) and increased lymphatic absorption (Palin *et al*, 1982). The role of the lipid digestion process will depend on whether the drug is administered with a dietary source of lipid or by presentation of the drug within a lipid-based formulation. Also, some of the components of lipid formulation, such as co-solvents and surfactants, may influence the lipid digestion process abrogating the advantage to drug bioavailability which is dependent on digestion of lipid (Pouton, 1985).

Class	Solubility	Permeability	IVIV expectation [1]	Potential [2] Microemulsion system	Anticipated Drug Delivery Benefits
I	High	High	IVIVC if the dissolution rate is slower than the gastric emptying rate , otherwise limited or no correlation	W/O	stabilization and protection against chemical and enzymatic hydrolysis
II	Low	High	IVIVC expected if the *in vitro* dissolution rate is similar to the *in vivo* dissolution rate, unless the dose is very high	SEDDS, O/W	improved solubilization and dissolution increased bioavailability
III	High	Low	Absorption (permeability) is rate determining and limited or no IVIV with dissolution rate	W/O	stabilization and protection against chemical and enzymatic hydrolysis increased bioavailability
IV	Low	Low	Limited or no IVIVC expected	SEDDS, O/W	improved solubilization and dissolution increased bioavailability

Table 1.1 The BCS for oral drug delivery and the potential microemulsion systems based on the aqueous solubility and membrane permeability considerations ([1]Amidon *et al*, 1995, [2]Constantinides *et al*, 1995)

1.2 Types of dietary lipids

In the Western diet, dietary fat constitutes a significant source of calories, approximating 30% of total caloric intake. Therefore, dietary intake of fat has received considerable attention in the last few decades since lipid food enhances the absorption of some hydrophobic drugs, such as cyclosporin A (Gupta *et al*, 1990). Carey *et al* (1970) has classified lipids into two main groups illustrated in Figure 1.1. Lipids were classified according to their interaction with water as either polar or non-polar lipid. Non-polar lipids are insoluble in the water phase *e.g.* cholesteryl esters (CE), hydrocarbons, and carotene (Figure 1.1A). The polar lipids are sub-classified further (Figure 1.1B): (I) insoluble non-swelling amphiphiles (vegetable oils, triacylglycerol, diacylglycerol, non-ionised long chain fatty acids and fat soluble vitamins); (II) insoluble swelling amphiphiles like (phospholipids); and (III) soluble amphiphiles like (detergents). For class I lipids, the addition to water form a thin film of lipid molecules, termed a monolayer. However, Class II lipids form a monolayer on the surface of water. They have a unique ability to interact with water to form laminated lipid-water structures referred to as lamellar liquid crystals. In the liquid crystal phase, non-polar groups of lipid molecules face one another sandwiching water between the polar groups, referred to as 'swelling'. Class III lipids can be further classified into soluble amphiphiles with lyotropic mesomorphism, like the sodium salt of long chain FAs and those without. When soluble amphiphiles with lyotropic mesomorphism exceeds their CMC, monomers aggregate to form micelles. In aqueous solution, the polar group of the monomers face outside, and the non-polar groups face inside. Lyotropic mesomorphism refers to when materials such as sodium oleate forms an intermediate liquid crystal phase before forming micelles (Nördskog *et al*, 2001). However, soluble amphiphiles such as bile salt will go through the intermediate liquid crystal phase (Carey, 1970).

1.3 Luminal digestion of dietary lipid

The majority of dietary lipid is ingested in the form of triglycerides, which are long chain length, C_{16}-C_{18} (Tso, 1985). Lipolysis is the result of three sequential steps which involve (I) dispersion of fat globules into a coarse emulsion of high surface area, (II) enzymatic hydrolysis of fatty acid esters (primarily triglycerides) at the oil/water interface and (III) dispersion of the products of lipid digestion by bile into an absorbable form allowing absorption into the portal vein or the lymph (Charman *et al*, 1996). Lipid digestion and absorption, including all the previous steps, is required for the conversion of triglycerides into a useful energy source (Carey *et al*, 1983).

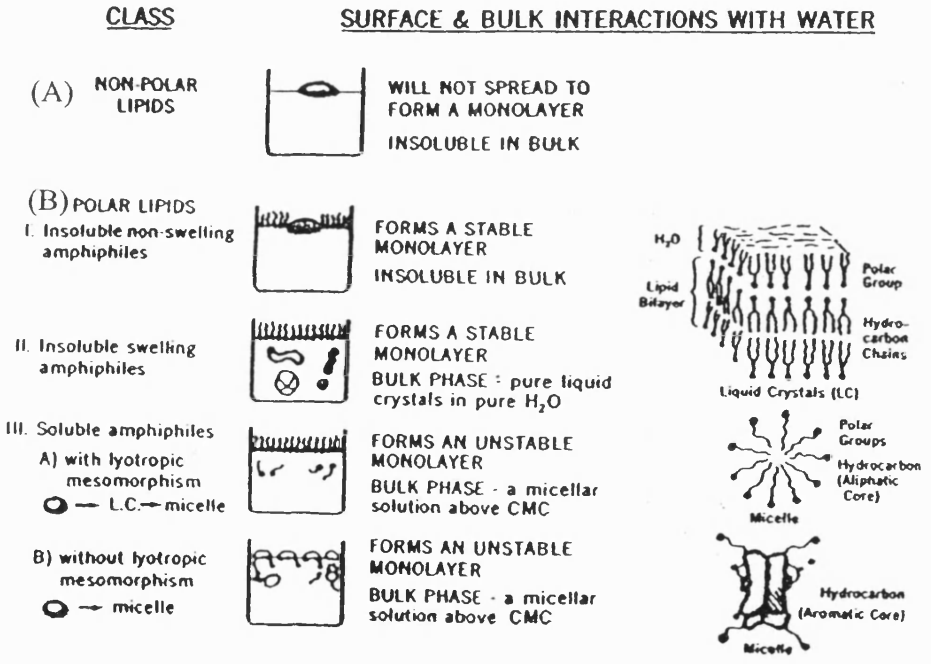


Figure 1.1 Classification of dietary lipids based on their interaction with water (Carey *et al*, 1970; Nördskog *et al*, 2001).

1.3.1 Digestion of lipids in the stomach

Primary lipid emulsification occurs in the stomach where human gastric lipase (hGL) has been proposed to be responsible for between 10-30% of total gastrointestinal lipolytic activity. Human gastric lipase has been found to be located inside granules in the apical region of chief cells and is mainly active in the fundic portion of the stomach (Moreau *et al*, 1989). Bodmer *et al* (1987) has cloned hGL and reported that it is a terminal glycoprotein of 379 amino acids with a predicted molecular weight of 43162. A 96 molecule region around Ser 152, includes the active site of pancreatic lipase (Gargouri *et al*, 1989;Winkler *et al*, 1990). Human gastric lipase was cloned from a partial N-terminal sequence obtained using a sample purified by gel filtration from human gastric juice. It has the ability to cleave the ester bond at the sn-1 and sn-3 positions of the TG, although the gastric lipase preferentially cleaves the sn-3 position relative to the sn-1 regardless of the FA. The activity of hGL is dependent upon triglycerides chain length (Gargouri *et al*, 1986b) and pH, the maximum activity reported to be at pH value 5.4 (Hamosh, 1984). Milk fat, the primary dietary source for neonates, contains considerable amount of SC and MC-TG. Both lingual lipase and gastric lipase play a major role in lipid digestion, particularly in neonates (Tso *et al*, 1982). Furthermore, the pancreatic lipase system is not fully developed in neonates.

In the normal diet, triglycerides and lecithin proportions (50:1 to 20:1) tend to form an emulsion system consisting of triglyceride droplets surrounded by an outer closed packed monolayer of lecithin (Carey *et al*, 1983) The lecithin monolayer decreases interfacial tension, so helping to stabilize the emulsion by preventing droplet coalescence and increasing the hGL activity. In addition, partially digested protein, complex polysaccharide and membrane-derived phospholipids present in the stomach, aid emulsification properties and can decrease surface tension further thus enhancing hGL activity (Carey *et al*, 1983). *In vitro* studies have shown a correlation between hGL activity and interfacial tension at the triglyceride - water interface

(Gargouri *et al*, 1984,1986a).

Inclusion of amphiphiles such as salts and some protein enhanced hGL activity by reducing interfacial tension. This was thought to be the result of protein unfolding due to different of the enzyme structure to denaturation (Solomon, 1998).

Products of lipolysis, which are diglycerides and fatty acids, have advantages in further enhancing emulsification by either lowering surface tension of triglyceride droplets (Hamsh *et al*, 1984) or by promoting immediate pancreatic lipase activity upon release of the emulsion into the duodenum (Gargouri *et al*, 1986). However, the mechanical forces of the stomach antrum (Armand *et al*, 1994) are thought to be important factor. Contractions increase intraluminal pressure so that the stomach contents are pushed towards the closed pylorus and then retropelled back to the stomach body, providing mixing followed by applied shear force, which tear the liquid interface apart when chyme is released through the pylorus (Solomon, 1998).

Short-and medium chain fatty acids in water exhibit pK_a values in the range of 4.8. However, because they are hydrophilic they are soluble in both ionised and unionised forms. Both are released as a result of hGL in the stomach and these fatty acids leave the surface of the fat droplets and are absorbed passively by the stomach mucosa. These are transported to the liver bound to albumin *via* the portal vein. Since the aqueous pK_a values of long chain fatty acids released by lingual as well as gastric lipases are at or above neutrality, they will be protonated at stomach pH values and form liquid fatty acid oils (Carey, 1983a). The squirting of antral contents back to the corpus provides most of the mechanical action involved in the initial emulsification of dietary TG. The DG and FA resulting from the action of acid lipases in the stomach, and the PL that is normally present in the diet, further aid the emulsification of dietary fat (Nördskog *et al*, 2001).

1.3.2 Duodenal digestion

In the duodenum, the enzyme responsible for the majority of triglyceride hydrolysis is pancreatic lipase, representing 2-3% of the total protein present. Some hGL activity will remain whilst the pH of the chyme is between 5 and 7 (Dutta *et al*, 1982). The lipid emulsion (chyme) enters the small intestine as fine lipid droplets (<0.5 μm) that are extremely stable (Carey, 1983; Tso, 1985).

Pancreatic lipase activity is optimal in the presence of a sufficient concentration of colipase, a non - enzymatic protein cofactor, (Borgström *et al*, 1984). Pancreatic lipase enzyme has a pH -activity profile opposite to the site of action in the gut. It has maximum catalytic activity in the pH range of 6.5-9.0 [cf. the duodenal pH range of 6.2-8.1 reported by Hernell *et al*, 1990] (MacGregor *et al*, 1997). Pancreatic lipase (L) binds to the substrate (S) from the hydrophobic site, but is rapidly inactivated by conformational changes in the absence of bile salt, as shown in Figure 1.2A. In addition, colipase binds to the interface and to lipase. The binding of colipase to lipase increases its activity by a factor 1.4-1.5, and stabilizes the enzyme (Figure 1.2A).

In contrast to hGL, the presence of amphiphiles such as bile salts or phospholipid at the surface of the emulsion droplet prevents binding of pancreatic lipase to the triglyceride surface (Carey *et al*, 1970) as demonstrated in Figure 1.2B. The mechanism is not yet well defined, but is most probably a surface effect and pH dependent (Borgström, 1975). Colipase acts in conjunction with the enzyme (in a molar ratio of 1:1) in order to protect pancreatic lipase from desorption induced by physiological concentration of bile salts, (reviewed by Pouton & Embleton *et al*, 1997) when bile salts are present above their critical micellar concentration. The presence of long chain fatty acids already in the core of the emulsion droplet from the previous hGL activity enhances binding affinity of colipase and thus pancreatic lipase to the lipid interface (Patton *et al*, 1978). Adsorption of the complex at the site of the triglyceride-water interface and the subsequent hydrolytic activity of pancreatic lipase

releases the lipolytic products, Sn-2 monoglycerides and fatty acids (Stadler *et al*, 1995). Diglycerides located at the lipid interface from previous hGL activity are also subject to further hydrolysis by pancreatic lipase. Positional specificity is an important factor. The enzyme shows a preference for Sn-1 and Sn-3 ester bonds. It is proposed that hGL has limited Sn-2 activity (Tiruppathi *et al*, 1982) and so can be differentiated from colipase- dependent pancreatic lipase. Pancreatic lipase acts more efficiently with 1-MG than with 2-MG. However, since the absorptive rate is comparatively fast, most of the 2-MG is absorbed before degradation or isomerization to form 1-MG (Nördskog *et al*, 2001).

Pancreatic lipase is secreted together with a proenzyme of colipase (procolipase) from acinar cells in the pancreas. Procolipase is activated to form colipase (Larsson *et al*, 1981) by trypsin cleavage, resulting in removal of the 5 amino acids from the N-terminus. The function of procolipase cleavage is unclear, as both procolipase and colipase are capable of promoting lipase-mediated hydrolysis of triglycerides (Larrson *et al*, 1991), though colipase was found to be more efficient than procolipase under certain reaction conditions.

Crystallography has been used to study the binding and activation sites of human pancreatic lipase at a lipid interface. It is defined that hPL is a single chain glycoprotein of 449 amino acids arranged as two domains connected by a hinge region (Winkler, 1990). According to the resolution of 3-D structure of hPL by X - ray crystallography, residues 337-449 form a carboxy terminal domain, which contains a binding site for the procolipase.

Normally a 1:1 stiochiometric complex forms between enzyme and cofactor. Procolipase has three finger shaped-regions with non-polar amino acids concentrated in each fingertip. These were thought to provide a means of binding between procolipase and the lipid interface.

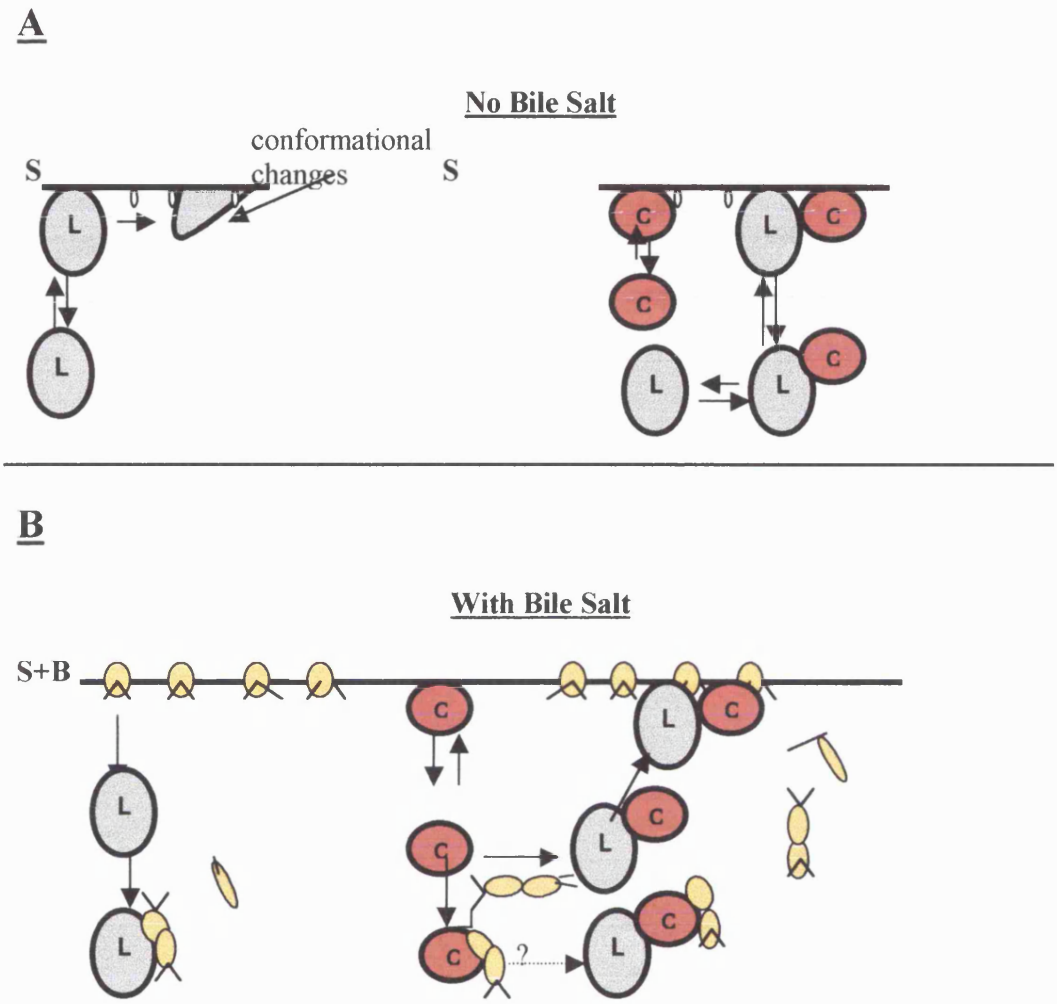


Figure 1.2 Representative model for the function of the lipase-colipase-bile salt system (Borgström, 1977b).

Residues 1-336 of hPL form the amino terminal domain (Winkler *et al*, 1990) typical of a α-hydrolyase fold (Olli *et al*, 1992) with a dominant central parallel sheet that contains the active site. The catalytic site is amino acids residues [Ser 152, Asp 176, and His 263 (Winkler *et al*, 1990)] with Ser 152 being the nucleophilic residue

essential for catalysis (Luthi-Peng *et al*, 1992). Conformational change of the enzyme is assumed to be necessary to allow contact with the active site, which is otherwise protected by an amphipathic helix acting as a lid (Luthi-Peng *et al*, 1992). The hydrophobic catalytic centre is uncovered due to rolling back of the lid, which is modulated by the hydrophobicity of the surface surrounding the active site (Brozowski *et al*, 1991).

The active site bound by phospholipid is apparently not changed, although the 3-D structure a hPL-procolipase complex, co-crystallized with mixed micelles of phosphatidylcholine and bile salts, indicates conformation changes in the enzyme cofactor complex (van Tilburgh *et al*, 1993). When the enzyme is in the closed conformation, van der Waals bonds exist between a surface loop (α-loop) over the active site and the lid. When the lid rolls back, the α-5 loop folds into the core of the protein rendering the active site serine, in the base of a hydrophobic canyon, accessible to the solvent ideal for lipid substrate binding.

The same study (van Tilburgh *et al*, 1993) reported that repositioning of the lid and α-5 loop in hPL also results in creation of an electrophilic region. This is an oxyanion hole around the active site, which acts to stabilize the negative charge generated during ester hydrolysis of the substrate. An interaction occurs between the N-terminal region of procolipase and the open conformation of the lipase lid via direct H-bonds, giving increased stability to the hydrophobic surface around the active site in preparation for interaction with the lipid interface. The importance of lid binding for function of the complex was illustrated by the chemical modification of procolipase residues involved in these H-bonds, which resulted in loss of procolipase activity (Larsson *et al*, 1991).

The bending angle between the C and N terminal domains of hPL has also been suggested to be important for positioning of the lid and subsequent catalytic activity (Aoubala *et al*, 1995). There is information regarding the possible interactions between substrate and enzyme made possible by crystallisation of hPL colipase

complex with an inhibitor of lipase (a C_{11} alkyl phosphate).

1.4 The physico-chemical state of lipids during digestion

The physicochemical behaviour of lipids and their hydrolytic products in the gastrointestinal tract is an area of interest that has attracted attention over many years (Hoffman *et al*, 1964). Major studies of fat digestion in humans were reported by Borgström (1974); Carey *et al* (1983); and Hernell *et al*, (1990). These workers reported *in vitro* studies of the equilibrium phase behaviour of relevant materials over a range of physiological relevant conditions. The emulsification of dietary fat is thought to occur mostly in the stomach, because no further marked emulsification has been observed in the duodenum, despite the presence of excess amphiphiles (Armand *et al*, 1999).

According to *in vivo* data, partially emulsified droplets in the stomach are between 1 to 100 μm diameter in the stomach, with the largest fraction in the range of 20 to 40 μm, while in the duodenum the emulsified droplets between 1 to 50 μm in size (Armand *et al*, 1994). Although studies of droplet size revealed the same size in the stomach and duodenum, droplets differed in lipid composition owing to the higher lipolytic activity in the duodenum. This was reflected in neutral lipid depletion and duodenal droplet enrichment with the lipolytic products, cholesterol and phospholipids (Armand *et al*, 1999).

1.4.1 The role of biliary lipids in drug dissolution and solubilization

Biliary lipids (bile salts and lecithin mixed micelles and cholesterol) are released as bile from the gall bladder and adsorb onto the surface of the crude emulsion, leading to the production of a more stable emulsion, with a smaller particle size. The reduction in droplet size provides an increase in surface area available for binding of the pancreatic lipase/co-lipase complex an interfacial enzyme system that acts at the surface of the emulsified TG droplets, leading to the quantitative production of one

molecule of 2-monoglycerides (MG) and two FA molecules for each TG (Shiau, 1981).

During digestion, a multilamellar liquid crystalline bilayer is formed at the oil water interface comprised mainly of monoglycerides and fatty acids, with phospholipid, cholesterol and bile salt (Charman *et al*, 1997). Parts of the liquid crystalline bilayer are released as a multilamellar vesicle due to the high surface pressure that results from the continued lipolytic activity. By the action of unsaturated bile salt micelles, layers from the multilamellar vesicles bud off and become solubilized to form unilamellar vesicles with hydrodynamic radii of between 400 to 600 Å. These represent the initial phase in the process of lipolytic product dispersal, being composed of aqueous mixed lipids saturated with bile salts. The formation of smaller intestinal mixed micelles (hydrodynamic radii of 80 Å) depends on the mixed lipid to bile salts ratio. The components of intestinal mixed micelles are bile salts, long chain fatty acids, monoglycerides, cholesterol and phospholipids. They act as a sink for lipolytic products under normal conditions *in vivo* (Semeriva *et al*, 1971).

1.5 Absorption of lipids

The absorption of lipophilic products (monoglycerides, medium and long chain fatty acids) usually occurs by passive diffusion through the brush border membrane (BBM) of the intestinal mucosa into the enterocytes. The BBM of the enterocytes lining the small intestine is separated from the bulk aqueous phase of intestinal contents by a poorly mixed unstirred water layer (UWL) that constitutes a major barrier to absorption of poorly water-soluble lipid digestion products. (Westergaard *et al*, 1974). The UWL is a near stagnant layer of water, mucus, and glycocalyx adjacent to the intestinal wall that is created by incomplete mixing of the luminal contents near the intestinal mucosal surface (Thomson *et al*, 1993).

Some evidence suggests that a certain amount of absorption is also possible directly from the unilamellar vesicular phases (Narayanan *et al*, 1996). This is a controversial

hypothesis which needs some examination. There is other evidence suggesting that absorption may be enhanced by the presence of a BBM fatty acid - binding protein (FABPm) in the cytoplasm of the epithelial cells (Stemmel *et al*, 1985). FABPm has been located on the outer surface of the enterocyte membrane and is thought to act as a medium and long chain fatty acid receptor (Clark *et al*, 1989), possibly helping lipid transport into the cell by acting as translocase (Nunn *et al*, 1986).

In this model, dissociation of the lipolytic product from the vesicles or micelles is necessary before absorption can occur (Westgaard *et al*, 1974). The mechanism of transfer of lipolytic products from the micellar phase to the BBM is thought to occur by one of three mechanisms (reviewed by Thomson *et al*, 1993). The collision model describes partitioning of the fatty acid directly from the micelle into the BBM of the enterocytes (Westgaard *et al*, 1974).

In the second model, the fatty acids first partition into an intermediate aqueous phase, UWL which is rich in solublizing materials and in which fatty acids are sufficiently soluble to promote diffusion and absorption by passive transport. This is referred to as "the aqueous" model (Thomson *et al*, 1993). An acid microclimate may be present within the UWL adjacent to the BBM (Levitte *et al*, 1992). Such a microclimate can be formed by mucus entrapment of hydrogen ions transported into the intestinal lumen by various types of antiporters (Blair *et al*, 1975, Lucas, 1984).

Fatty acid uptake may occur *via* the "dissociation" model, which depends on an acid microclimate (reviewed by Thomson *et al*, 1993). The unionised form of fatty acid is ensured by the low pH of this environment, leading to decreasing solubility in micelles and enhancing partitioning into BBM (Shaiu, 1990). These results increased critical micelle concentration (CMC), enhancing dissociation of micelles and promoting fatty acid release (Shaiu, 1981). Bile salts can act repeatedly until lipid absorption is complete, because upon dissociation of the intestinal mixed micelles the bile salt diffuses back to the intestinal lumen where once again it can form micelles, which take up further lipolytic product (Solomon, 1998). Most bile salts are actively

absorbed in the terminal ileum, then transported to the liver and enter the enterohepatic circulation (Lewis *et al*, 1990).

1.6 Lipid metabolism within enterocytes and lymphatic transport.

There does not appear to be a clear explanation in the literature regarding the mechanism by which the lipoidal products move across enterocyte cell membrane (Embleton *et al*, 1997). Once in the enterocyte, long-chain fatty acids and monoglycerides are assumed to diffuse through the cell cytoplasm to the endoplasmic reticulum, and intracellular fatty acid-binding proteins detected in enterocytes may be involved (Cistola *et al*, 1989). Lipolytic products are then reassembled into triglyceride.

Two pathways of triglycerides synthesis have been identified (reviewed by Tso, 1985). The extent to which each pathway is utilized depends upon the lipid mixture absorbed. The quantitatively important or default pathway is the monoglyceride path, illustrated in Figure 1.3, and which accounts for 85% of intestinal triacyglyceride synthesis. This involves the direct acylation of absorbed, exogenous 2-monoacylglycerol with activated fatty aids via MG acyl transferase (MGAT) to form corresponding diglycerides and subsequently *via* diacylglycerol acyltransferase (DGAT) to form TG (reviewed by Porter *et al*, 2001). If the monoglyceride supply is low, the α-glycerophosphate (the phosphatidic acid) pathway converts three molecules of absorbed fatty acids and endogenous glycerol into triglycerides within microsomes (Thomson *et al*, 1993). The phosphatidic acid pathway appears to be more important in the re-esterification of endogenous fatty acids because it uses the endogenous glycerol-3-phosphate and therefore is not dependent on the absorbed monoglycerides (Tso *et al*, 1987).

The source of the lipid core for assembly of lipoprotein (Thomson *et al*, 1993), namely chylomicrons (CM) (through the monoacylglycerol pathway) and very low-density lipoprotein (VLDL) (through the phosphatidic acid pathway), are the re-

esterified triglycerides with acyl chains of more than 12 carbon atoms (Morre, 1977). The chylomicrons and very low-density lipoprotein particles are spherical complexes consisting of a triglyceride core surrounded by a coat of cholesterol, phospholipid and apoprotein. Both are the primary lipoproteins secreted by the intestine. Under fasting conditions where intestinal lipid concentration is low, VLDL appears to be dominant, whereas CM production increases at higher lipids intake (Tso *et al*, 1987). These complexes stabilize lipid in the aqueous medium of the cytoplasm and lower the cellular concentration, promoting passive absorption. Complexes migrate toward the basolateral membrane and are released by exocytosis into the intercellular space (Nunn *et al*, 1986). This directs them to the lymphatic system *via* the lacteal ducts and they then pass through the thoracic duct into the left subclavian veins. Subsequently they are taken up by the liver and adipose tissue (reviewed by Couvreur *et al*, 1996).

The short or medium chain triglycerides (less than 12 carbon atoms) do not take part in re-esterfication of triglycerides; rather, they partition into the continuous phase of the gut lumen and are absorbed passively. They are formed in the stomach mucosa, conjugated with albumin in the portal vein and transported to the liver (Staggers *et al*, 1981).

In summary, the intestinal bilayer is one of the least permeable membranes in the body and is known to contain FA transporter proteins (Cortot *et al*, 1978). Both cytosolic fatty acid binding proteins, I-FABP and L-FABP play an important role in the intracellular transport of absorbed FA. FABP exhibits a degree of specificity in lipid migration to the endoplasmic reticulum and the subsequent re - esterfication process, since FABP prefers binding to long-chain fatty acids. The absorption of lipophilic molecules from the intestinal lumen is considerably more complicated than was first thought.

Recent data suggest that I-FABP can directly extract fatty acid from membranes (Thumser *et al*, 2000) and bind fatty acids presented to the apical side of the

enterocyte more than those presented basolaterally, indicating that I-FABP may be more important in the delivery of fatty acid from the brush border membrane to specific intracellular sites. Another hypothesis indicated that more fatty acid appears to be bound to L-FABP in the enterocytes (Alpers *et al*, 2000) and studies in mice and Caco-2 cells suggest that I-FABP is not necessary in the intracellular processing of fatty acids. An open mind needs to be kept on the mechanisms by which drugs can be absorbed from solubilized systems.

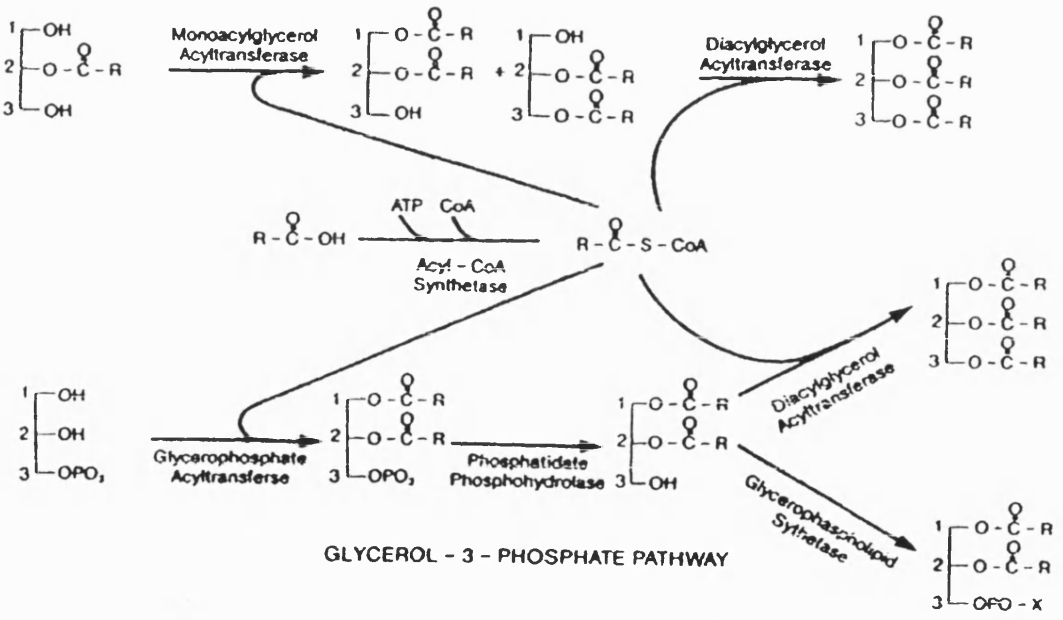


Figure 1.3 Schematic representation of the α- Monoacylglycerol pathway.

1.7 Role of the lipid digestion process in drug absorption from lipid based formulation

Assuming that absorption of a poorly-water soluble drug can be enhanced by lipid processing, it is possible to speculate about the formulation factors which will affect absorption. Different mechanisms by which stimulation of the lipid digestion cascade

may enhance drug bioavailability are discussed below.

1.7.1 Gastric emptying

The bioavailability of formulations of poorly soluble drugs could be improved by lengthening the residence time in the gastrointestinal tract allowing complete dissolution (reviewed by Couvreur *et al*, 1996). Recent research has revealed mechanisms by which contact time can be extended by careful adjustment of the timing of dosing, taking into consideration normal physiological activities, like gastric emptying.

The stomach functions as a mixer and grinder, regulating the rate of delivery of gastric contents into the intestine. Wall tone following a fatty meal is affected by some of the lipolytic products released by hGL activity. These are emptied from the stomach as spurts of 1-5 ml into the duodenum, reducing the rate of gastric emptying (Cortot *et al*, 1982).

Cholecystokinin, a polypeptide hormone, is involved in slowing gastric emptying (Schwizer *et al*, 1997). The presence of fat in the terminal ileum slows the rate of gastric emptying and therefore co-administration with a fatty meal or postprandial administration may enhance the absorption of poorly soluble drugs, by increasing the residence in the stomach and upper intestine (reviewed by Couvreur *et al*, 1996).

There is extra literature describing the possible benefits of reducing gastric emptying time, which can prolong the drug absorption phase and, hence, improve drug bioavailability. Recent work reported a 95% increase in bioavailability of ontazolast when administered as a 20% soybean oil-in-water emulsion over that obtained from an aqueous suspension. The improved bioavailability was due to prolonged absorption of ontazolast from the emulsion formulation resulting from slower gastric emptying. With ontazolast there was a need for pre-absorptive lipolysis of triglycerides (Soybean oil) prior to release and subsequent gastrointestinal absorption of ontazolast (Hauss *et al*, 1998). The oral absorption of cefoxitin from arachis oil

and Miglyol 812 has been investigated in rats. In this case, pre-dosing the animals with Miglyol 812 before administration of Cefoxitin in an aqueous suspension did not enhance absorption, suggesting that increased drug absorption may not be due to slowing gastric emptying. Enhanced absorption with Miglyol emulsion, compared with the arachis oil emulsion, was still obtained following intraduodenal administration, which also indicating that gastric emptying was unimportant (Palin *et al*, 1986).

Griseofulvin (Crounse, 1961), phenytoin (Hamaguchi *et al*, 1993), and danazol (Charman *et al*, 1993) have a greater bioavailability in the presence of food. One possible explanation is that delayed gastric emptying caused by the presence of food in the stomach allows more time for disintegration and dissolution of these formulations before they enter to the small intestine (Mithani *et al*, 1996). It is, however, difficult to isolate this as the causative mechanism.

1.7.2 Emulsification

The physiological emulsification process, which occurs in the stomach and continues during lipolysis, may have a beneficial effect on the dispersion of lipid formulations, resulting in formation of a larger surface area available for drug release and enzymatic activity. In general, dispersion of lipid droplets to form an emulsion of high surface area is an essential step in the efficient intestinal absorption of lipids. Ontazolast, as discussed before, was investigated using four different formulae, an intravenous solution, aqueous suspension, emulsion (a 20% soybean oil in water emulsion with Tween 80®), and a SEDDS composed of Gelucire 44/14® and Peceol®.

The SEDDS forms a very fine emulsion on contact with the GI fluid *in vivo*. Peceol® is a readily dispersible lymphotropic solubilizing agent comprised mainly of a mixture of a mono-and di-glycerides of oleic acid. Peceol® has chemistry similar to the end - products of intestinal lipid digestion. In this study the SEDDS was chosen with reference to previous studies and showed a significant increase in the absorption

of the hydrophobic drug, cyclosporin A, from "pre-digested" olive oil, when compared with non-digested oil.

It was proposed that lymphatic transport of many lipophilic drugs occurs in association with triglyceride absorption from the gastrointestinal tract. Thus, it was hypothesized that Peceol® would provide an efficiently absorbed source of lipid for promoting lymphatic drug transport. In fact, the relative ontazolast bioavailability determined from the $AUC_{(0\text{-}8\%)}$ calculated from plasma profiles, did not differ significantly for any lipid-based formulation. Compared with the emulsion, variability was somewhat less for the SEDDS 20/80 and Peceol solution, suggesting that the variable cause of lipid digestion may have reduced gastrointestinal absorption of Ontazolast from the emulsion. The values of C_{max} were variable and did not differ significantly between any of the lipid-containing formulation but followed the rank order of SEDDS 20/80> emulsion > SEDDS 50/50> Peceol® solution. However, the values of T_{max} were reduced and similar for both SEDDS formulation in comparison to Peceol® solution and the emulsion formulation, for which T max values were over 2-fold greater.

From this study, it can be concluded that, although Peceol® represented more closely the end-stage of lipid digestion, the Ontazolast absorption from Peceol® solution represented by the T_{max} was identical with that of the emulsion. This suggests that the absorption of Ontazolast from Peceol® is dependent on factors other than a reduced need for absorptive lipolysis. The addition of Gelucire 44/14® (HLB=14) to Peceol® (HLB=3) increased the HLB of the final SEDD formulation. This has been reported to facilitate the release of hydrophobic drugs from lipophilic vesicles and to facilitate more efficient formation of a fine dispersion of the solubilized drug in the GI fluids.

1.7.3 Digestion

Perhaps a better strategy for the formulator is to design the lipid formulation to act as a suitable substrate for lipolytic enzymes. The production of a colloidal solution by

the lipid digestion process has already been discussed. Another factor in favour of digestible lipids is the stimulatory effect the lipolytic product would have on gall bladder evacuation, enhancing bile salt and phospholipid secretion in high concentration with cholesterol (Carey, 1983).

Bile salts and lecithin possess surfactant properties summarized in Table 1.2, which enhance the dissolution rate of crystalline drugs by reduction in surface tension and increased wettability of the surface of the drug. The contact angle between solid surface and a liquid can used to express the wetting properties of bile salts (Florence *et al*, 1986). The closer the contact angle of the liquid to zero, the greater the wetting of the solid, whereas values 90° or above are known prevent liquid penetration into capillary pores of a powder and inhibit wetting (Saunders, 1971).

Particle size reduction of the drug enhances dissolution. However, hydrophobic drugs may agglomerate upon fine particle reduction depending on the shape of the resultant powder aggregates. The pore size and contact angle will influence the ability of liquid to penetrate the powder capillary and wet the solid surface (Bates *et al*, 1966).

Bile salts can increase the rate of dissolution *via* enhanced wetting in a drug-specific manner. Miyazaki *et al.* (1979,1981) suggested that the enhanced dissolution of phenylbutazone in simple bile salt solutions was mediated via increased wetting, whereas micellar solubilization was responsible for improved dissolution of indomethacin. Similarly, Weintraub *et al* (1969) found that the effects of physiological bile salt concentrations could not be explained by solubility results alone.

Solubilization of hydrophobic drugs is defined as passage into solution of substances that are hydrophobic in a given medium of bile salts, involving the previous presence of a colloidal solution whose particles take up and incorporate within or upon themselves (McBain *et al*, 1955). Micelles are aggregates of surfactant monomers

(when present above the CMC), which are capable of rapid breakdown and reformation. Solubilization of insoluble materials may occur upon partitioning within micellar structures. There is sufficient evidence in the literature to support the view that bile salt-lecithin mixed micelles are usually a good solvent, for lipophilic drugs (reviewed by MacGregor *et al*, 1997). However, the mechanism by which the dissolution rate is enhanced may vary (Baskatselou *et al* 1991, Naylor 1993) *in vitro*, bile salts enhanced dissolution for hydrophobic compounds that exhibit food related increase in absorption such as griseofulvin (Crounse, 1961), danazol (Martha *et al* 1982, Miyazaki *et al* 1979) and phenytoin (Miyazaki *et al* 1981). However, BS do not enhance dissolution in all cases, such as with large molecular weight antibiotics· pafenolol, neomycin, and kanamycin, penicillamine and tetracycline (reviewed by Charman *et al*, 1997).

	Mechanisms
1	Solubilization/Wetting
2	Prolonging GI residence time (decreases gastric emptying, decreased GI motility.
3	Protection from luminal degradation
4	Protection form brush border or luminal metabolism.
5	Enhanced membrane permeability
6	Increased membrane contact/absorption by lipid adsorption mechanism
7	Absorption via lymphatics
8	Decreased hepatic secretion.

Table 1.2 The mechanisms by which lipids and surfactant increase oral bioavailability.

Dissolution is usually described by the Noyes-Witney equation (Weintraubs *et al*, 1969)

$$DR = \partial c/\partial t = (Ac_S D)(Vh) \qquad \text{eq 1.1}$$

DR, the dissolution rate, is a function of the surface area available for dissolution, A, the saturation solubility of the compound, D, the volume of the dissolution medium, V, and the boundary layer thickness, h.

As well as the physical features of the drug, many physiological parameters can also play a role in determining the dissolution rate (Dressman *et al*, 2000). The physical and physiological parameters relevant to drug dissolution are tabulated in Table 1.3, along with their corresponding parameters in Eq. (1.1)

Parameter	**Physical factor**	**Physiological factor**
Surface area	Particle size	Native surfactants
Diffusion coefficient	Molecular size	Viscosity of the luminal content
Boundary		Motility patterns
Layer thickness		Flow rates
Solubility	Hydrophilicity	pH, buffer capacity
	crystal structure	**Bile, food components**
Concentration of drug in solution		Permeability
Volume of GI contents		Secretions, Administered fluids

Table 1.3 Physical and physiological parameters affecting class II drugs dissolution

The dissolution rate of hydrophobic drugs is often increased by the action of bile salts. Bile salts may decrease interfacial energy between the drug and the dissolution medium, thereby increasing the effective surface area (A) available for dissolution (O'Driscoll, 1996). They may also increase the solubility of the drug (Cs) *via* micellar solubilization. Typically, wetting effects predominate at bile salt concentrations below the CMC and solubility effects tend to prevail above the CMC (Heertjes *et al*, 1969/1970).

However, *in vivo* results using bile duct-cannulated rats indicated that the presence of endogenous bile the enhanced absorption rate of ethyinylestradiol-3-cyclopentylether from the small intestine. In summary, the mechanisms by which bile salts enhance dissolution rate and solubility of poorly soluble drugs has been shown to be a combination of wetting, solubilisation and the physico-chemical properties of the drug (Couvreur *et al*, 1996). For a series of structurally-related steroid compounds, the wetting effect was reported to be predominant over solubilisation effect of bile salts. However, for danazol, the most lipophilic compound studied, the increase in solubility was the most important factor (Couvreur *et al*, 1996).

Several workers have taken the step of using lecithin and bile salts in a micellar system. A further consideration is that lecithin is converted by phospholipase A2 into lysolecithin in the duodenum. Lysolecithin enhanced dissolution rates and solubilisation of hexestrol, dienestrol, and griseofulvin in *in vitro* systems (Bates *et al*, 1967). Hydrophobic bile salts form aggregates or micelles when their concentration exceeds the critical micellar concentration (CMC). Different bile salts form different types of micelles and the structure depends on the concentration of bile salts (Schwartz *et al*, 1996). The CMC of different bile salts can change as a function of the pH and is influenced by the concentration of lipids and cations. For example, trihydroxy bile salts have a higher CMC than dihydroxy bile salts. At physiological pH, the CMC of most bile salts varies between 2 and 5mM (Schwartz *et al*, 1996). Concentrated bile exists as mixed aggregates in the form of vesicles or micelles when stored in the gall bladder.

Originally, Small (1967) described the structure of bile salt/lecithin mixed micelles as disc-shaped, bimolecular leaflets of lecithin surrounded on their hydrophobic parts by bile salts molecules, as shown in Figure 1.4A (Carey, 1983). Later, Mazer *et al* (1980) proposed the mixed disc model in which the disc-shaped micelle was proposed to comprise a lecithin bilayer (possibly with cholesterol and/or cholesterol ester incorporated) interspersed at constant ratio with hydrogen bonded bile salt

dimers. Some bile salts molecules were arranged as a layer on the outer micelle surface, where they prevented contact between the lecithin hydrocarbon chain and water, as presented in Figure 1.4B.

Early studies, suggested that, at physiological bile salt:lecithin ratios (< 2:1), the structure of the micelle is "an isometrical particle of globular shape, having a centrosymmetric arrangement" *i.e.* lecithin molecules are sandwiched between a half circle of bile salt dimers (reviewed by Naylor, 1993), as shown in Figure 1.4C. Recently, studies reported by Walter *et al* (1991) and Kozlov *et al* (1997), the vesicle - micelle transition of PC-NaTDC was assessed by comparing cryo-transmissoin electron microscopic (Cryo-TEM) images of the structures formed with the associated turbidity changes. They identified an intermediate stage between vesicles and small spheroidal mixed micelles, and showed that, at the upper boundary, all the structures are spheroidal mixed micelles, as shown in Figure 1.4D.

The initial step in vesicle solubilization at low cholate concentration is associated with pore formation and recognition of the bilayer lipid. The region of transient pore formation is followed by the formation of larger sheets (twenty to several hundred nm in diameter), where their hydrophobic edges are stabilized by cholate molecules. The high edge activity of cholate induces the formation of cylindrical mixed, flexible micelles that appear to peel from the bilayer sheet. These structures are similar to the so-called 'rod-like' structures proposed by Hjelm *et al.* (1988,1990) and are not like the mixed disc model previously described by Small (1970). Commonly, cylindrical micelles are expected to form close to the micellar phase, which is close to a phase boundary with pre-micellar bile salt/lecithin vesicles (Pedersen *et al*, 1995).

1.7.4 Assessment of drug and lipid absorption

Enhancing absorption of hydrophobic drugs by alteration in membrane permeability in the presence of lipolytic product, has become a wide area for discussion (Muranishi, 1985) (Table 1.2). Mixed micelles could enhance intestinal permeability

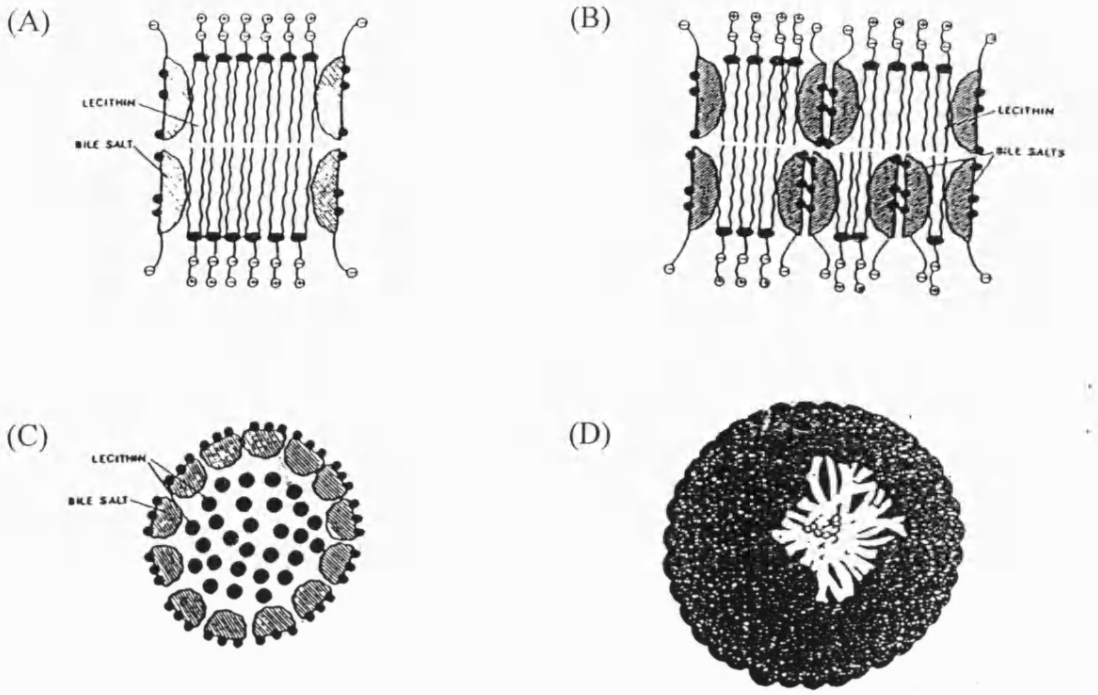


Figure 1.4 Proposed molecular models for the structure of bile salt-lecithin mixed micelles. (A) disc-shaped model, (B) mixed disc shaped model, (C) "an isometrical particle of globular shape, having a centrosymmetric arrangement" *i.e.* lecithin molecules are sandwiched between a half circle of bile salt dimers, (D) spheroidal mixed micelles (Carey, 1983; Naylor *et al*, 1993).

by three different processes:

Firstly, lipolytic product and BS may alter the intrinsic solvent properties of the intestinal membrane leading to increased penetration *via* paracellular or transcellular routes (Muranishi *et al*, 1994).

Secondly, solubilization of poorly water-soluble drugs within bile salt micelles may

facilitate diffusion through the UWL, leading to increased absorption. Mixed micelles, which facilitate solubilization of high quantities of lipophilic drug and enhance drug diffusion across the UWL, may release high concentrations into the aqueous fluid surrounding the microvilli ready for absorption across BBM. The UWL has been suggested to be the main barrier to intestinal absorption of lipophilic drugs. Components of mixed micelles and lipid-based formulations, *i.e.* bile salts amphiphiles, non-ionic surfactants and fatty acids, have been reported to increase membrane permeability (Curatolo *et al*, 1994) by altering the configuration of the membrane in such way that it becomes easier for drugs to permeate (Muranishi, 1985). Increasing the bioavailability of the drug in the presence of lipid digestion products depends on the nature of the drug absorption. Highly polar and/or high molecular weight drugs may be improved by bile salts or other amphiphiles.

Thirdly, in spite of all the efforts to increase the absorption of hydrophobic drugs by different mechanisms, there are some reported disadvantages, which may affect intestinal absorption. Some fatty acids increase the thickness of UWL and reduce the intestinal blood flow. Micellar solubilization of drugs may result in reduction in the rate of diffusion of free drug whilst retention of drug within micellar structures may reduce free drug available for absorption (Poelma *et al,* 1991, del Estate *et al,* 1993). However, the ability of single, specific lipid components to enhance lymphatic transport, namely, degree of saturation, fatty acid chain length and lipid class, have the predominant effect on the bioavailability of lipophilic drugs.

1.7.4.1 Degree of unsaturation of lipid

In general, the degree of fatty acid unsaturation has been shown to have a large effect on the rate of absorption and partitioning of lipids between portal blood and intestinal lymphatics. Lipids with increasing degrees of unsaturation appear to produce larger lymph lipoprotein particles and preferentially promote lymphatic lipid transport (Cheema *et al*, 1987, Okner *et al*, 1972). These early *in vivo* studies suggested that the improved absorption and intracellular transport of unsaturated lipids resulted from

both improved digestion and a more significant increase in lipid fluidity and corresponding decrease in lipid hydrophobicity at body temperature for unsaturated rather than saturated lipids (Cheema *et al*, 1987, Bergstedt *et al.*, 1990).

In vivo examination of the lymphatic transport of palmitic acid, oleic acid and the trans-isomer of oleic acid, elaidic acid, found that administration of oleic acid led to re-esterification *via* the monoglyceride pathway and secretion of chylomicrons. In contrast, the fully saturated palmitic acid and elaidic acid resulted in higher phospholipid output, in the form of smaller lipoproteins, possibly via stimulation of the α-glycerophosphate pathway (Bernard *et al*, 1987). In support of these general trends, Noguchi *et al* (1985) reported a doubling of the lymphatic transport of testosterone undecanoate after administration in a polyunsaturated (MaxEPA) lipid vehicle when compared with monounsaturated oleic acid, in spite of subsequent studies showing that the extent of lymphatic transport of MaxEPA oil ($C_{20:5}$) and olive oil (primarily $C_{18:1}$) were similar (Chernenko *et al*, 1989).

1.7.4.2 Chain length of the administered fatty acids

The partitioning of absorbed (and potentially resynthesised) lipids between the portal blood and the intestinal lymph was first described in the 1950s. In these studies, the majority of fatty acids with chain lengths of C_{14} and above were found to be recovered in thoracic lymph, whereas a larger proportion of the shorter chain lipids were absorbed directly into the blood (Bloom *et al*, 1951a, 1951b).

Sylven and Borgström (1969) demonstrated that the lymphatic transport of exogenously administered cholesterol increased in a linear fashion after co-administration with triglycerides of increasing fatty acid chain length. Vahouny and Treadwell (1958) described similar trends such that the lymphatic transport of cholesterol was increased by co-administration with oleic acid, linoleic acid and stearic acid, although, in these studies, no increase in cholesterol transport with butyric, lauric or palmitic acid was seen. Furthermore, of all the respective

triglycerides, only trilinolein improved the lymphatic transport of cholesterol above that seen with a control (lipid free) vehicle.

Similar fatty acid chain length effects have been observed in the absorption and lymphatic transport of co-administered drugs. For example, in fasting human volunteers, vitamin D3 was more efficiently absorbed after administration in peanut oil® (a long-chain triglyceride containing primarily oleic acid) than in Miglyol 812® (a medium-chain triglyceride containing primarily C_8-C_{10} saturated fatty acids). Since vitamin D3 is almost exclusively absorbed by the lymphatic route, the low absorption observed when administered with the medium-chain triglyceride probably represents a reduction in lymphatic transport, secondary to insufficient chylomicron formation (Holmberg *et al*, 1990). The effect of saturated fatty acids was dependent on the carbon chain length; the C_8 caprylic acid had less effect than the C_{12} lauric acid and C_{16} palmitic acids. Cefoxitin absorption was enhanced in the presence of lauric acid as a result of a reversible transient effect on the membrane fluidity and not by complex formation between cefoxitin and the fatty acid. Medium chain fatty acid could also increase permeability of cefoxitin by fluidity the membrane (Palin *et al*, 1986).

A recent study was done by Caliph *et al* (2000) using Hf in cannulated, consicous, unrestrained rats after administration in lipidic vehicles with different fatty acid chain lengths. Both lymphatic transport (C_{18}-based vehicle, 15.8% of dose> $C_{8\text{-}10}$, 5.5%> C_4, 2.22%>C_0, 0.34%) and total systemic exposure (C_{18}, 22.7% of dose> $C_{8\text{-}10}$, 19.2%> C_4, 15.2%>C_0, 6.4%) of Hf were enhanced by the presence of lipids in the formulation specifically by an increase in the fatty acid chain length of the co-administered lipid. Increase in lymphatic drug transport appeared to correlate with increases in lymphatic lipid transport (Caliph *et al*, 2000).

1.7.4.3 Glycerides structure of administered lipid

The absorption of structured lipids has also been well studied (reviewed by Tso *et al*, 1999; Porter *et al*, 2001) and it is clear that the variability in the structure inherent in the design of these systems may improve drug delivery systems. Charman and Stella (1986) examined the lymphatic transport of DDT in anaesthetized rats and reported a 2-fold increase in the cumulative extent of lymphatic transport of DDT after administration in a formulation comprising long-chain (C_{18}) fatty acid compared with the equivalent triglyceride-based lipid vehicle. A shorter lag time was also associated with administration in the fatty acid vehicle when compared with the triglyceride (TG) vehicle. They thought that this was a result of the shorter time required for the synthesis of chylomicrons from the fatty acid vehicle when compared with the TG vehicle that required additional pre-absorptive digestion.

In contrast, Charman and his colleagues (2000) recently examined the lymphatic transport of halofantrine (Hf), after administration to conscious rats in simple solutions comprising either oleic acid/glyceryl monoolein (FA / MG) or peanut oil (TG) and no significant differences in the extent of lymphatic transport of Hf were evident (Porter *et al*, 1996). This may due to a compound specific difference, but also represent the potential differences in processing power of the GIT between the two studies.

In the DDT study, the lipid solutions were administered intraduodenally to anaesthetised rats, whereas the Hf formulations were administered orally to conscious rats. It is possible; therefore, that the increased luminal processing (and, specifically, pre-duodenal, gastric processing) improved oral dosing in combination with the possible suppression of intestinal mixing in anaesthetised animals. This led to a more complete processing of the TG formulation in the Hf study, circumventing the advantages seen previously with the pre-digested FA/MG formulation in the DDT experiments. However, the impact of various structured lipids on the absorption of co-administered compounds such as cholesterol is much less clear (Tso, 1985;

Porter *et al*, 1996) and their application as pharmaceutical excipients has not been widely addressed.

1.7.5 Intestinal lymphatic absorption of hydrophilic drugs

Co-administration of lipids (or food) has been shown to increase the bioavailability of poorly water-soluble drugs (Winstanely *et al*, 1989, Toothacker *et al,* 1980, and Armstrong *et al*, 1980). The lymphatic system present throughout the gastrointestinal tract varies in terms density, absorptive capacity and functional characteristics. The major physiological role of the intestinal lymphatics is in the transport of fats, lipid soluble vitamins and to aid in the maintenance of the body`s water balance. The intestinal lymph, along with hepatic and regional lumbar lymph, drain *via* cysterni chyli into the thoracic lymph duct which empties into the general circulation at the junction of the left internal jugular and left subclavian veins (Charman *et al*, 1991). Hydrophobic compounds transported by the intestinal lymphatics are almost exclusively associated with the triglyceride cores of chylomicrons synthesised by the enterocytes (Hauss *et al*, 1998).

The major factor contributing to significant lymphatic drug transport is the co-administration of a suitable lipid source to " drive " chylomicron synthesis (Hauss *et al*, 1998). Chylomicrons are triglyceride-rich lipoprotein particles and range in size between 50 and 500 nm. A typical composition is 86-92% triglycerides, 6-8% phospholipid, 2-4% sterol (free and esterified) and about 1-2% protein (Charman and Stella, 1991). Chylomicrons are present in lymph at concentration of nearly 1-2% during periods of peak lipid transport (Tso *et al,* 1982, Shiau *et al,* 1985).

In 1951, Bloom *et al*, found that the factors which determined transport of fatty acids were either the free form through the portal blood of intestinal lymphatics or re-esterified to a triglyceride (reviewed by Charman & Stella, 1991). The portal blood transport of orally administered fatty acids appears to increase as the chain length of the fatty acid decreases (as mentioned before).

Long-chain fatty acids are re-esterified to corresponding triglycerides that are then incorporated into, and transported by chylomicrons. Medium or short- chain fatty acids are poorly transported by the lymphatic system, as they are not incorporated to any extent in chylomicrons (Noguchi *et al*, 1985). Biochemical findings support this hypothesis because of the specificity of the fatty acid binding protein for long-chain fatty acids (Ockner *et al*, 1974), and the inactivity of acyl:CoA synthase towards fatty acids of chain length less than 12 carbons (Dawson *et al*,1960).

While the lipid-based formulation significantly increased the amount of ontazolast (discussed earlier) transported by lymph, the total amount was insufficient to account for the improvement in bioavailability. Ontazolast had maximum lymphatic transport from the emulsion and Peceol® formulations, which are the highest rates of concurrent triglyceride transport. However, the SEDDS formulations, which enhanced absorption of ontazolast from the gastrointestinal tract, resulted in the highest concentration of ontazolast in the chylomicron triglycerides.

Based on to this study, the authors suggested that the fraction of absorbed drug transported via lymphatics is influenced by (I) the rate of drug absorption from the gastrointestinal tract and (II) the rate of concurrent triglyceride transport (Hauss *et al*, 1998). The intestinal lymphatic route is a specialised absorption and transport pathway for highly lipophilic drugs, lipidic derivatives and lipophilic xenobiotics.

Kwei *et al* (1998) studied the lymphatic transport of a poorly water-soluble (<1 g/ml) and highly lipid-soluble (>80 mg/ml in soybean oil®) compound: 5 - reductase enzyme inhibitor, MK-386, from three oral formulations: an aqueous suspension, a mixed lipid/surfactant (Imwitor®, mono- and di-glycerides of capric and caprylic acids/polysorbate 80) formulation and a long-chain lipid (soybean oil) solution formulation after oral administration of radiolabelled MK-386 to rats. Interestingly, the rank order of the total amount of MK-386 transported into the mesenteric lymph over a six hour post-dosing period was: aqueous suspension>mixed lipid/surfactant system>soybean oil, *i.e.* greatest for the lipid-free formulation. Furthermore, the

mixed lipid/surfactant formulation contained a relatively large amount of surfactant, notably in excess of the critical micelle concentration and this may have affected the rate and extent of dissolution and subsequently the absorption profile of the poorly water-soluble MK-386. Nevertheless, MK-386 was primarily transported to the systemic circulation by the intestinal lymph (after administration of all three formulations), with up to 80-fold higher levels seen in the lymph compared with the portal blood.

One strategy for drug delivery, associated with intestinal lymphatic transport of lipophilic drugs is the potential for by-passing hepatic first pass metabolism after oral dosing and/or targeting drugs to regions of the lymphatic system. Applications of monoglycerides appear to hold promise for selective lymphatic delivery. These prodrugs are designed to avoid pre-systemic clearance, reduce gastrointestinal intolerance, and for targeting various disease states resident within the intestinal lymphatics (Charman et *al*, 1996).

Recently a triple-cannulated conscious dog model, which allows sampling of thoracic duct lymph, portal and systemic blood, has been described (Khoo *et al*, 2001). In contrast to the rat model, the dog model allows administration of dosage forms that are of a size relevant for human administration, and facilitates study of the effects of fed versus fasted states in drug absorption. In rat this is not possible as, due to the lack of a gallbladder, bile flow is continuous and the pulsatile response to food which occurs in dogs and man is absent (reviewed by O'Driscoll, 2002).

Postprandial administration of halofantrine (Hf), an important antimalarial, leads to 3- and 12-fold increases in oral bioavailability in humans and beagles, respectively, and corresponding 2.4-fold and 6.8-fold decreases in metabolic conversion to desbutylhalofantrine (Hfm). Factors contributing to the decreased postprandial metabolism of Hf could include inhibition of presystemic CYP3A metabolism by food components and/or recruitment of the intestinal lymphatics as an absorption pathway. Although previous rat studies confirmed Hf base is a substrate for

lymphatic transport, it is difficult to extrapolate such data to higher species, as the largely constant bile flow in a rat precludes attainment of representative pre- and postprandial states, and formulations administered to rats are often not relevant to higher species. These limitations have now been addressed by development of a conscious dog model that allows simultaneous study of intestinal lymphatic and non-lymphatic drug absorption and aspects of enterocyte-based drug metabolism. After oral administration of 100 mg Hf base, the mean fasted and postprandial lymphatic transport was 1.3% and 54% of the administered dose, respectively. Comparison of portal and systemic plasma Hfm concentration profiles suggested enterocyte-based conversion of Hf to Hfm; however, the proportion of Hf metabolized to Hfm was similar after fasted or postprandial administration. Hence, it appears that the previously observed decrease in the postprandial metabolism of Hf is largely a consequence of significant postprandial intestinal lymphatic transport (which bypasses first pass hepatic metabolism). This new dog model will facilitate identification of the key factors that impact bioavailability, lymphatic transport, and metabolic profiles of highly lipophilic drugs (Khoo *et al*, 2001).

1.8 Drug Delivery systems f or poorly-water soluble Drugs

Although lipids have been used in galenicals for over 100 years (Groves *et al*, 1976), the commercial success and clinical acceptance of lipid-based formulations for significant drugs with large market values has only occurred within the last two decades (Pouton, 1982; Wakerly *et al*, 1986).

As the commercial introduction of new lipid-based formulation is expected to increase, the question which arises is what is driving the growing interest in lipid-based formulations? Some of the reasons for the increasing interest in lipid-based systems include the following: (i) an improved understanding of the manner in which lipids enhance oral bioavailability and reduce plasma profile variability, (ii) new drug candidates being less water soluble and more potent (thereby allowing for lower unit doses), (iii) the higher purity and better characterisation of lipidic excipients, (iv)

formulation versatility, (v) possible metabolic and biopharmaceutic advantages (Charman, 1999).

Lipid systems offer great potential in the design and fabrication of new carrier systems. Not only do lipids form a wide variety of physical structures, as seen in Table 1.4, but also each has the potential to solubilize varying quantities of poorly water-soluble drugs, hence achieving molecular dispersion of many drugs whose bioavailability might be comprised by poor dispersion characteristics (Florence, 1997).

Micelles
Emulsion w/o;o/w;w/o/w;o/w/w and o/o
Microemulsions
Self-emulsifying system
Vesicles: Liposomes; noisome
Vesicles-in-water-in-oil systems
Organogels
Bicontinuous systems

Table 1.4 Varieties of lipidic systems.

1.8.1 Classification of lipid delivery systems

A key strategy to better utilization of lipid-based formulations is to consider their performance from both formulation and physiological standpoints (Charman, 1999). The physico-chemical factors determining the absorption potential of an individual drug molecule are well known, and are shown in Table 1.2.

"Lipid formulations" have evolved from simple water-immiscible solvents to important microemulsions, that may potentially improve the bioavailability of poorly water - soluble drugs through induced changes in the absorptive environment of the small intestine. Hoar and Schulman introduced microemulsion as early as the

1940s.by generating a clear single-phase solution by titrating a milky emulsion with hexanol.

Microemulsions are 'thermodynamically stable, isotropically clear dispersions of two immiscible liquids, such as water and oil, stabilized by an interfacial film of surfactant molecules' (Constantinides *et al*, 1994). The surfactant may be pure, a mixture, or combined with other additives. Ordinary emulsions differ from microemulsions in that they require a large energy input, are thermodynamically unstable and will eventually separate.

In 1982, Pouton proposed a new type of lipid formulation termed 'self-emulsifying drug delivery system' (SEDDS), typically composed of 30% of Tween 85® and 70% of Miglyol 812® (saturated medium and long chain fatty acids). In 1986, Wakerly produced a similar formulation consisting of 30% Tagat TO® (polyoxyethylene-(25)-glycerol trioleate) and 70% Miglyol 812®. The self-emulsifying behaviour of the Tween 85® was found to be sensitive to acidic conditions; therefore, the Tagat TO® system is considered more useful.

Self-emulsification is a phenomenon which has been used commercially for many years in formulations of herbicides and pesticides (Groves, 1978). In early decades, these formulations were diluted to produce concentrates of crop-sprays allowing very hydrophobic compounds to be transported. On the other hand, self-emulsifying drug delivery systems (SEDDS) using safe excipients and delivered via the oral route have recently been used to improve the lipophilic drug dissolution and absorption of compounds such as cyclosporin A, ritnovair, saquinvair (Charman *et al*, 1997).

SEDDS are isotropic mixtures of natural or synthetic oil, solid or liquid non-ionic surfactant, and/or co-surfactant (Charman *et al*, 1992). They form fine oil-in-water emulsions when introduced into aqueous media with mild agitation (Kommur *et al*, 2001). It is useful to note that under the definition given, SEDDS are not microemulsions, although they may be considered to be very close to

microemulsifying systems when added to aqueous systems (Lawrence *et al*, 2000). SEDDS formulation offers many promising features for hydrophobic drugs; therefore, there is a need for sub-classification to aid the interpretation of comparative bioavailability studies.

Pouton (1999) classified SEDDS using three main criteria by which lipid formulation can be distinguished: 1) Does the formulation self-emulsify or remain poorly dispersed in water? 2) Is the dispersed formulation digestible by lipases? 3) When the formulations make contact with water are some of the components lost by dissolving in the aqueous phase? There are three useful types of formulations which typically have such properties and are shown in Table 1.5.

The simplest of these, non-emulsifying systems Type 1, contain a drug dissolved in an easily digestible oil usually made up of vegetable oil or medium chain-length triglycerides (fractionated coconut oil). These are safe food substances, categorized as GRAS (generally regarded as safe) by regulatory agencies and do not present a toxicological risk to the formulator. These preparations work well, as glycerides are rapidly digested *via* lipolysis into 2-monoglycerides and free fatty acids, which are then solubilized within bile salt-lecithin mixed micelles to form a colloidal solution.

A reservoir of drug is likely to be formed in the colloidal dispersion, as the hydrophobic drug should solubilize in the mixed micelles, from where the drug can partition to be absorbed effectively by the passive (transcellular) route (MacGregor *et al*, 1997). Oily formulations may be an option for potent compounds with log P >4 and their simplicity is appealing to formulators. For example, the bioavailability and the plasma profile of 5 α-reductase inhibitor from an oil solution were equivalent to these achieved using a self-emulsifying system (Loper *et al*, 1996). When an appropriate dose of the drug can be dissolved, a Type I formulation may well be the system of choice, in view of its simplicity and biocompatibility. However, the low drug solvent capacity of most oils, combined with their poor miscibility with the aqueous environment of the GIT leading to variable drug absorption, limits their use (Amemiya *et al*, 1998; Pouton, 2000).

Type II, self-emulsifying formulations contain water-insoluble components including triglycerides, mixed mono- and di-glycerides and lipophilic surfactants (HLB < 12). The addition of a lipophilic surfactant may improve the solvent capacity of the formulation. Surfactants act to reduce the tension at the oil/water interface promoting micellar formation. As a result, these vehicles produce very fine dispersions in the GIT, with particle sizes ranging between 100-250 nm in systems consisting of polyoxyethylene-(25)-glyceryl trioleate (Tagat TO®) and medium chain triglycerides (Wakerley *et al*,1986). This is a large surface area for drug partitioning which leads to improved drug absorption, independent of digestion, increasing bioavailability (Charman *et al*, 1992, Lawrence *et al*, 2000).

The main feature of Type II is (a) efficient self-emulsification and (b) absence of water-soluble components. Typically, Type II systems are best formulated with medium chain triglycerides and/or mono- or di-glycerides, mixed with ethoxylated oleate esters with HLB values of approximately 11 (water-dispersible surfactant) (Table 1.5) (Pouton, 1999). The minimum concentration of surfactant needed to form a self-emulsifying formulation is between 25%-40% with particle size 200-250 nm. In the presence of surfactant at 50%, the particle size will be approximately 100 nm. The efficiency of the emulsification is slower and contributed a viscous liquid crystalline gels, which form at the oil-water interface, when the concentration of the surfactant more than 65%. In such a case, very high energy is required to break up the particles to produce a stable emulsion. However, are no longer considered a self-emulsifying system.

The mechanism of interaction the Type II with the aqueous phase could be explained by the "diffusion and stranding". These water-dispersible surfactants (upon mixing with aqueous phase), strongly interact with water, and form a variety of structured liquid crystal phases, and tend to form W/O/W emulsions spontaneously. Pouton (1999) proposed that self-emulsification is closely linked to the formation of dispersed lamellar liquid crystal formation allowing penetration of water into formulation, resulting in interfacial disruption, which is sufficient enough to peel

emulsion particles from the surface of the bulk oil-surfactant interface. Type II systems will be digestible unless the surfactant concentration is too high. Solomon (1998) investigated the presence of the surfactant on the lipolysis process. It was reported that the surfactant are very slowly digested, and their presence at the oil-water interface can inhibit the digestion of glycerides present in the formulation. Surfactants with HLB >12 will inhibit the lipolysis of the glycerides.

Type III systems are similar to Type II but include hydrophilic surfactants (HLB > 12) and may include co-solvents, such as propylene glycol and ethanol. Co-solvents aid emulsification by diffusion and stranding, where they cause a disruption at the o/w interface by penetrating into the surfactant monolayer. This increases the distribution of lipophilic components in the aqueous phase. During mixing with excess water, the bulk of the hydrophilic, micelle-forming surfactant may also be transferred to the aqueous phase to form a micellar solution. The fate of the drug and lipid components after mixing with water could be solubilization in a swollen micellar solution, dispersion as a fine emulsion, or isolation and precipitation (Pouton, 1999; 2000).

These formulations generally have a greater drug solubilising capacity, although this may be reduced upon dilution in water (Constantinides *et al*, 1994). It is difficult to predict the phase changes that may occur on dilution and in practice these will occur dynamically, in a non-equilibrium manner, making it difficult to predict drug solubility and fate from the corresponding equilibrium phase-behaviour. Type III can be sub-classified into Type IIIA and Type IIIB according to the proportion of water soluble surfactants or co-solvents.

Type IIIA (Table 1.5) is typically mixtures of medium chain fatty acids oils, mixtures of mono-, di- and tri-glycerides and hydrophilic surfactant. Type IIIB, are similar to Type IIIA, with the addition of a water-soluble cosolvent. Because of the greater hydrophilic content in Type IIIB, these are likely to result in a colloidal solution of drug and oil in aqueous micelles. The motivation for using hydrophilic surfactants or

water-soluble co-solvents may be to increase the solvent capacity for drugs with intermediate log*P* (2<log*P*<4). This approach was used for reformulating cyclosporin A as 'Neoral' (Vonderscher and Meinzer, 1994). Type IIIB formulations present the highest risk of precipitation. The extent of precipitation will depend on the physical chemistry of the drug and how hydrophilic is the formulation. Type III can be referred to "self-microemulsifying systems" due to the optical clarity upon addition to the aqueous phase. Recently, Gershanik and Benita (2000) proposed a new type of SEDDS termed "lipid self-dispersing pharmaceutical vehicles" capable of forming drug-loaded fine lipid particles in the gastrointestinal (GI) lumen. These include self-microemulsion formulation, surfactant dispersions, pre-formulated freeze-dried, or microencapsulated emulsion and lipid/cross-linked polymeric matrices (reviewed by Gershanik *et al*, 2000). All these formulations can be administered in soft or hard gelatin capsules; and will produce fine oil droplets/micelles dispersion upon capsule disintegration and contact with aqueous solution. However, water-containing vehicles cannot be filled into soft gelatin capsules due to the hydrophilic nature of the gelatin shell, which absorbs water thus dehydrating and eventually disrupting the emulsion structure (Wakerly *et al*, 1986).

1.8.2 Biopharmaceutical issues

The choice of the formulation for each drug will depend on a number of factors; the required dose, on which types of formulation have sufficient solvent capacity to allow formulation of a unit dose, and in particular to the fate of the drug after the formulation has been administered to the gut. Most hydrophobic drugs will have low solubility in the lumen, so the target is to keep the drug in 'reservoir' form for absorption (Pouton, 1999).

Using the principle when simple monobasic electrolyte drugs are absorbed, the lumen becomes temporarily depleted of ionized drug and then a supply of free ionized drug (reservoir) available rapidly. If the base is strong enough such that the drug is

predominantly ionized in the small intestine, then dissolution of the solid drug in the intestine will be rapid and its overall solubility in the lumen will be high.

In the case of weak bases, ($pKa < 7$), they will not be highly soluble in the small intestine, may dissolve in the stomach, with a possibility of precipitation after gastric emptying into the small intestine. Therefore, weak bases will not get the benefit of a drug reservoir if they administered in solid dosage form, due the dissolution rate-limiting step. Consequently, weak bases may benefit from reformulation in lipids which can provide a reservoir of drug dissolved in either lipid or micellar solution. The drug will stay in solution through its passage into the gut.

At this stage, the formulator should balance the physico-chemical properties of the drug with the lipid formulation *i.e.* a balance is required to produce a low-volume unit dose formulation while avoiding precipitation of the drug in the gut.

In practice, some solvents could solubilize the drug *e.g.* co-solvents (PEG), which may satisfy the need to increase the drug content in each capsule but would lead to unwanted precipitation. The use of co-solvents is often said to result in an acceptable precipitation in the form of 'nanocrystalline 'suspensions, which would provide high surface area for dissolution (Liversidge *et al*, 1995). Whether precipitation can be controlled to form a nanocrystalline suspension from type IIIB products is unclear. To avoid drug precipitation, Type I and Type II formulations would be preferred to keep the drug stable until digested. However, this is not possible for all drugs (such as cyclosporin A) because of their limited solvent capacity. This limitation has initiated the use of Type III.

Generally, the most difficult drugs are those which have limited solubility in both water and lipids (typically with $\log P$ approximately 2). The rate of absorption is another issue that should be taken into consideration. Self-emulsifying systems are known to disperse rapidly within the contents of the stomach, so the formulation will empty into the intestine as the aqueous solution. As expected, the rate of absorption

of Type II and Type III systems are likely to be rapid, especially when given to a fasted stomach. Hypnotics or analgesics may benefit from this kind of formulation because they require a very rapid onset of action. However, such a formulation could be a disadvantage for a drug which has a low therapeutic index. Type I formulations could be safer for drugs that are intended for chronic use, both in view of the pharmacokinetic profile produced and also since they avoid the chronic administration of surfactant.

In contrast, Type II and Type III could be used for drugs that are to be administered for patients who have a compromised lipid digestion. The 'Sandimmune Neoral' formulation has the advantage over the early Sandimmune formulation because of the small particle size after dispersion facilitates rapid absorption. This is a significant advantage in the treatment of patients who lack full bile function, such as liver transplant patients (Amante *et al*, 1997). Small particle size is not crucial for good bioavailability in all cases.

The content of the capsule will be emptied into the digestive environment of the upper small intestine, so the most important factor may not be the size of the particles in the initial dispersion, but rather their susceptibility to digestion and/or solubilization by mixed micelles of bile salt and phospholipids. Therefore, reducing the oil content and including a surfactant and water-soluble co-solvent as in Type IIIB will decrease the possibility of droplets being digested. This suggests that self-emulsifying systems are dependent on the initial emulsification process to produce a colloidal dispersion (Pouton, 2000).

Increasing Hydrophilic Contents

	Type I	Type II	Type IIIA	Type IIIB
Typical composition				
Triglycerides or mixed glycerides	100%	40-80%	40-80%	<20%
Surfactant	0	20-60% (HLB<12)	20-40% (HLB>11	20-50% HLB>11
Hydrophilic co-solvents	0	0	0-40%	20-50%
Particle size of dispersion	Coarse	100-250nm	100-250nm	50-100nm
Significance of aqueous dilution	Limited Importance	Solvent capacity unaffected	Some loss of solvent capacity	Significant phase changes & potential loss of solvent capacity
Significance of aqueous digestibility	Crucial requirement	Not crucial but likely to occur	Not crucial may be inhibited	Not required not likely

Table 1.5 Classification of SEDDS formulations (Pouton, 1999).

1.9 Scope of the research

The scope of the present study can be summarized as follows:

(1) Investigation of a novel 'surfactant-free' lipid formulations containing oils, mixed glycerides and water-miscible co-solvents. These types of formulations use excipients which have GRAS status, which offers toxicological advantage. The solvent capacities of surfactant–free formulations have been compared with other lipid formulations.

(2) To study the effect of the physico-chemical properties of hydrophobic drugs and the fate of the drug after dispersion of the formulation in water or simulated intestinal fluids. New methods have been developed to determine the equilibrium solvent capacity of dispersed formulation.

(3) To examine the digestion of lipid formulations, in the presence of pancreatic lipase. A general method, which uses a pH-stat to determine liberation of free fatty acid, has been available for some time but, given the complexity of the gut lumen, there is still much to learn about the use of *in vitro models* of lipolysis. This study included a further examination of the variables which can affect lipolysis, and the significance of these variables on hydrolysis of long-chain triglycerides (LTG), medium-chain triglycerides (MCT), tributyrin (SCT) and mixed mono-and-diglycerides (MG).

(4) To explore the digestion of individual excipients and representative lipid formulations from each class.

(5) To investigate the influence of lipid excipients on phase separation following dispersion in simulated intestinal fluid, in the absence and/or the presence of pancreatic lipase and bile salts.

Chapter 2

An investigation of the potential of "Surfactant-Free lipid Drug Delivery Systems".

2.1 Introduction

There are some concerns regarding the use of surfactants in formulations, particularly for chronic administration. With an increasing number of lipid excipients available for use, there is a need for more information to allow the optimal choice of excipients. More knowledge is needed regarding surfactants, in particular of their toxicity and how important they are to the performance of a formulation.

One motivation for the formulator is to find an alternative formulation using lipid excipients which have GRAS (generally regarded as a safe) status. A study using rat intestinal preparations showed that 0.1% w/v aqueous solutions of both polysorbate and block copolymer non-ionic surfactants caused damage to the epithelial border (Walters *et al*, 1981). Upon removal of the non-ionic surfactant, the damaged epithelial border was readily repaired by the physiological regeneration of the gastrointestinal membrane, suggesting that the acute use of surfactant is likely to be tolerated.

This study addresses the short-term effects of absorption of a lipid formulation but, in many instances, chronic administration is required. Metabolism of ester ethoxylate type non-ionic surfactants in the gastrointestinal tract initially results in hydrolysis to release free polyoxyethylene glycol, fatty acids and the sorbitan or glycerol moiety from the backbone (Swenson *et al*, 1992). Due to their hydrophilicity and high molecular weight, polyoxyethylene glycols are not absorbed and are mainly excreted in the faeces. Such materials could solubilize cell membrane lipids, which may be a contributory factor to the observed membrane disruption.

The limited solubility of some drugs in lipidic solvents, uncertainties about what occurs to co-administered drugs/lipid in the gastro-intestinal tract and a lack of

predictive testing procedures, both *in-vitro* and in commercial production, goes hand-in-hand with the safety issues of lipid excipients (Pouton, 1997).

For this reason, formulations containing oils, mixed glycerides and water-miscible co-solvents, most of which have GRAS status, were investigated in this study. The conditions under which isotropic solutions are formed with oil, mixed glycerides and co-solvent, was investigated using a series of steroidal drugs and hydroxy benzoate derivatives (log *P* 1.3-4).

The objective was to investigate whether these formulations offer any extra solvent capacity over (Type I) oil and mixed glyceride blends. Steroidal and hydroxy benzoate derivatives were considered to be models of typical hydrophobic drugs. Solvent capacity of surfactant-free formulations were compared with other SEDDSs formulations. In order to determine the equilibrium solubility accurately, samples were pre-heated and left to equilibrate before determining their solubility. The data of these tests are shown in Appendix 1, and summarized in this chapter.

2.2 Materials

Co-surfactant (mixture of mono-, di-, and tri-glycerides)

Trade name	Scientific name	Dominant fatty acids	Molecular weight	HLB	Manufacture
Imwitor 988®	Mixture of caprylic glycerides	$C_{8:0}$ > 50 % $C_{10:0}$ > 40 %	300	4-6	Condea Chemie, GMBH Hüls , INC
Capmul MCM®	Mixture of caprylic and capric fatty acids	$C_{8:0}$ > 75 % $C_{10:0}$ > 10 %	343	5-6	Abitec Corp. (Coloumbus, OH)

Imwitor 988®

Structure is: R-O-CH_2-CH-OH-CH_2 -O-R

R = H or C_8, acyl radicals

Capmul MCM®

CH_2----------$OOCR_1/R_2$
|
CH----------OH +
|
CH_2----------OH
1- (or, α) Monoglycerides

CH_2----------OH
|
CH----------$OOCR_1/R_2$ +
|
CH_2----------OH
2- (or, β) Monoglycerides

CH_2----------$OOCR_1/R_2$
|
CH----------OH +
|
CH_2----------$OOCR_1/R_2$
1,3 - Diglycerides

CH_2----------$OOCR_1/R_2$
|
CH----------$OOCR_1/R_2$ +
|
CH_2----------OH
1,2 - Diglycerides

CH_2---------$OOCR_1/R_2$
|
CH----------$OOCR_1/R_2$ +
|
CH_2---------$OOCR_1/R_2$
Triglycerides

+ Glycerol

+ Free Fatty Acids

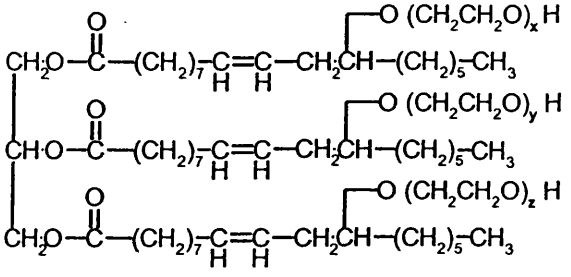
$R_1 = C_7H_{15}$; $R_2 = C_9H_{19}$

The RCO_2 moiety is predominately caprylate and caprate with trace amounts of caproate, laurate and higher molecular weight fatty acids.

Hydrophilic surfactant

Cremophor RH 40®: (USP name Polyoxyl 40 hydrogenated Castor oil).

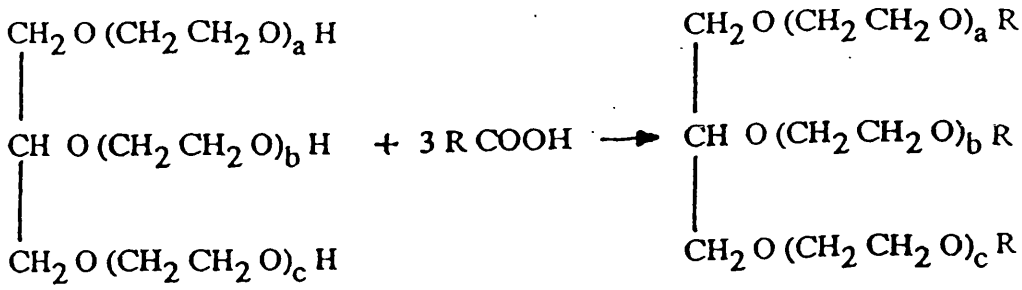
Cremophor RH 40® is made by reacting 45 moles of ethylene oxide with one mole of hydrogenated castor oil (with HLB value = 14-6). It consists of hydrophobic and hydrophilic regions. A hydrophobic part is formed by glycerol polyethylene oxystearate with fatty acids glycerol polyglycol esters. The hydrophilic region is formed by polyethylene glycols and ethoxylated glycerol.



The number of moles of ethylene oxide reacting per mole of castor oil is the sum of **x**, **y** and **z**, which in turn is expressed as the ethoxylation number. Cremophor RH 40 was obtained from BASF, Inc.

Tagat TO®:

Tagat TO® (polyoxyethylene- (25) -glycerol trioleate) is produced by the esterification of ethoxylated glycerol with oleic acid. A known weight of ethylene glycol oxide is treated with glycerol to produce polyethylene glycol glyceryl ether. The esterification is then continued using a commercial grade of oleic acid, obtained from hydrolysis and fractionation of olive oil. Tagat TO® contains an average of 25 ethoxy residues per molecule with HLB value of 11.3 and Mwt of 3400. It was supplied by Goldschmidt Chemical, Germany.



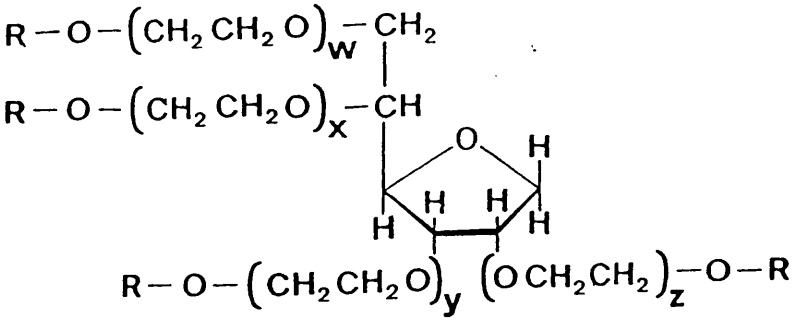
Fatty Acid POE-(XX)-GTO Surfactant

Where a+b+c=25 for Tagat To

R is principally $CH_3(CH_2)_7CH{=}CH\ (CH_2)_7\ COOH$

Tween 80® : polyoxythylene-(5)-sorbitan monoleate

Tween 80® is non-ionic surfactant belonging to a class known as polysorbate surfactants. It is manufactured from a nucleus with six hydroxyl groups. The hydrophilic character of Tween 80 is produced by introducing polyoxyethylene chains at hydroxyl sites. The molecular weight is 1310 and HLB value is 15. It was purchased from Sigma, UK.



Medium chain Oils

Miglyol 812®: Fractionated vegetable fatty acids of mixture of decanoyl ($C_{8:0}$) and octanoyl ($C_{10:0}$) glycerides with a molecular weight of 550. It was provided from Condea Chemie. Gmbh, (Hüls, Inc).

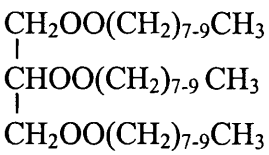
The fatty acid content is shown below:

FA C_6 < 0.1

FA C_8 < 56.9

FA C_{10} < 42.2

FA C_{12} < 0.7



Water-soluble co-solvents:

Transcutol P®: Ethoxydiglycol (INC)

Purified diethylene glycol monoethyl ether (EP). It is manufactured from raw materials of petrochemical origin, with Mwt of 134.17. It was supplied by Alfa Chemical Ltd, (Gattefossé, France)

Chemical formula: C_2H_5-O-CH_2-CH_2-O-CH_2-CH_2-OH

PEG 400:

Poly(oxy-1,2-ethandiyl), Mwt = 400, Aldrich, UK.

Propylene glycol:

Propane-,1,2,3-triol, Mwt = 76.9, Sigma, UK.

Glycerine:

1,2,3,propanetriol, Mwt = 92.09, Sigma, UK.

Solvents.:

Ethanol 96% v/v GPR.

Methanol 96% v/v GPR

*Both solvents were acquired from BDH Chemicals (Dorest, UK).

Drugs :

Table 2.1 *Steroids*

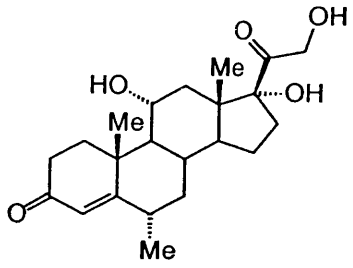
Name	Chemical formula	Log P	Mwt	Cas. #	Supplier
Testosterone	$C_{19}H_{28}O_2$	3.3	288.4	T 1500	Sigma
Progesterone	$C_{21}H_{30}O_2$	3.8	314.7	P 0130	Sigma
Hydrocortisone	$C_{21}H_{30}O_5$	1.53	362.47	H 4001	Sigma
Testosterone Acetate	$C_{21}H_{30}O_3$	4.7	330.5	T 1625	Sigma
Hydrocortisone Acetate	$C_{23}H_{32}O6$	2.48	392.49	H 4126	Sigma

Table 2.2 Hydroxy benzoate

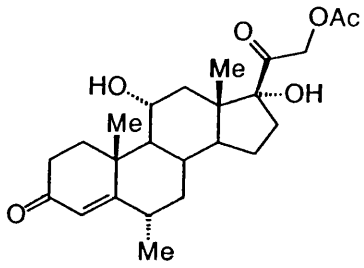
Name	Chemical formula	Log P	Mwt	Cas. #	Supplier
Butyl paraben	$C_{11}H_{14}O3$	3.24	194.2	H 9503	Sigma
Methyl paraben	C_8H_8O3	1.66	152.1	H 6654	Sigma
Ethyl paraben	$C_9H_{10}O3$	2.19	166.18	11,198-8	Aldrich
Propyl paraben	$C_{10}H_{12}O3$	2.71	180.2	P 5835	Sigma

Steroids

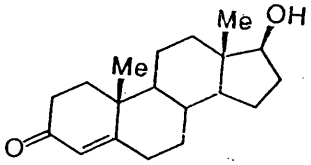
Hydrocortisone



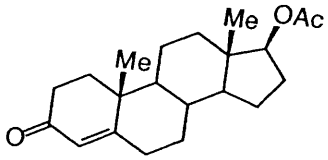
Hydrocortisone acetate



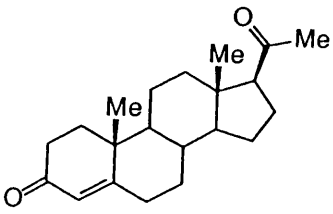
Testosterone



Testosterone acetate

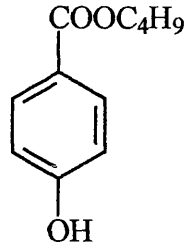


Progesterone

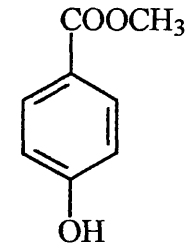


Hydroxy benzoate derivatives:

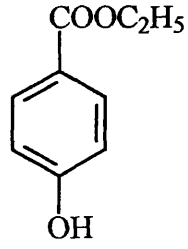
Butyl paraben



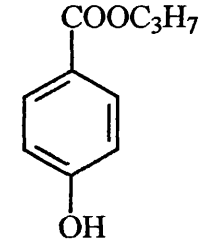
Methyl paraben



Ethyl paraben



Propyl paraben



2.3 Methods

2.3.1 Surfactant-free formulations / Phase behavior

Formulations were classified into two main categories, as shown in Table 2.3. Those which included surfactant and those which are surfactant-free. The focus of the initial part of this investigation was surfactant-free formulations prepared from tri-, di- and mono-glycerides with water-soluble co-solvents. The initial objective was to determine regions of mutual solubility using phase diagrams. Each of the axes on the diagram represented the percentage contributed to the formulation by each of the three constituents. For phase studies, 5 g mixtures of the medium chain triglyceride of fractionated coconut oil (Miglyol 812®) plus the co-surfactant (mixture of mono-, di-, and tri-glycerides; Capmul MCM® or Imwitor 988®) were mixed with water–soluble co-solvent in 20 g screw-capped vials using a 1 ml Gilson pipette. The three components of the system were thoroughly mixed by using a vortex mixer (Whirlimix, Fisons Ltd), placed in water bath at 50°C for 2 mins, then kept for 24 - 48 hour at room temperature at 25°C before being visually assessed for phase behaviour. Six combinations of triglyceride, medium chain fatty acid oil and water-soluble co-solvent were selected to provide a range of possible formulations. If the mixture of constituents formed a continuous phase throughout the vial, the formulation was classified as miscible, whereas vials which displayed two or more phases were described as immiscible. For systems comprising four components, the weight ratio of a pair of hydrophilic co-solvents was kept constant so that there were only three variables, each of which can be presented by one side of the triangular phase diagram.

2.3.2 Equilibrium solubility studies

2.3.2.1 Determination of equilibrium solubility at 50°C and 25°C

The equilibrium solubility was determined by pre-heating samples at 25°C and 50°C. The samples were composed of steroids dissolved in surfactant-free formulae

comprising mixtures of tri-, di- and mono-glycerides and water-soluble co-solvents. Once the miscible surfactant–free formulations have been identified, bulk mixtures as shown in Table 2.4 were prepared by ad-mixing appropriate quantities of various components and mixing with a vortex mixer (Whirlimix) or stirring if necessary to ensure thorough mixing. Once a stable, clear, formulation had been prepared, the drug of interest was added with vortexing for 4 minutes to ensure mixing, with three replicates prepared for each sample. The samples were heated for 1.5 hour at 50°C then equilibrated at 25°C with continuous shaking. The samples were analyzed using UV spectrophotometer analysis method at 0, 6, 12, 24, 36, 48 hours, 4, 6 days and 2, 3, 4 weeks to ensure that the solubility at equilibrium had been determined.

2.3.2.2 Determination of the equilibrium solubility of testosterone at different temperatures.

Solubility studies in viscous oils present two problems. At 25°C, the dissolution may be slow so that equilibrium is not reached. If the oils are heated to get the drug into solution, then excess drug may not re-crystallize. Testosterone was used as a model drug to examine the effect of several temperature of initial solubilization on the apparent solubility after equilibration at 25°C. This was a further check that the solubility at equilibrium was being accurately estimated. Samples were prepared as in section 2.3.2.1, heated at different temperatures (30, 40, 50, 60, 70 and 80°C) then stored at room temperature with continuous shaking. The pre-heated samples were then analyzed after intervals of time of 0, 6, 12, 36, 48 hours and 4 days at 25 °C.

2.3.3 Solubility studies of drugs in lipid formulations

Once the miscible surfactant–free formulations have been identified, bulk mixtures were prepared admixing appropriate quantities of various components and mixing with a vortex mixer (Whirlimix) or stirring if necessary to ensure thorough mixing. Once a stable, clear, formulation had been prepared, the drug of interest was

Formulation	Hydrophilic co-solvent	Triglyceride	Lipophilic co-surfactant
1	PG	FCO	Imwitor 988®
2	PEG400	FCO	Imwitor 988®
3	Transcutol P	FCO	Imwitor 988®
4	Gly.+PG	FCO	Imwitor 988®
5	Gly.+Trans.P	FCO	Imwitor 988®
6	Glycerine	FCO	Imwitor 988®

Table 2.3 Combinations of triglyceride, hydrophilic co-solvent and lipophilic co-surfactant selected as possible formulations

	Increasing Hydrophilic content →				
Composition	Type I	Type II	Type III A	Type III B	Surfactant-Free
*Triglycerides or mixed glycerides	100%	40-80%	40-80%	< 20%	50-80%
*Surfactant HLB <12	0	20-60%	0	0	0
HLB >11	0	0	20-40%	20-50%	0
*Hydrophilic cosolvents	0	0	0-40%	20%-50%	20%
*Particle size of dispersion	coarse	100-250nm	100-250nm	50-100nm	< 200nm
*Significance of aqueous dilution	limited mportance	solvent capacity unaffected	some loss of solvent capacity	significant phase changes potential	solvent capacity affected
*Significance of digestibility	crucial requirement	not crucial but more likely to occur	not crucial but may be inhibited	not required not likely to occur	more likely to occur

Table 2.4 Surfactant – free formulations compared to SEDDS formulations (Modified from Pouton, 1999).

added, vortexing for 4 minutes to ensure mixing. The three replicates were prepared for each sample. The formulas were subsequently stored at room temperature for 48 hours for equilibration before assay.

2.3.3.1 Solubility of steroids and hydroxy benzoate derivatives in lipid excipients.

In order to understand more clearly the solubility of steroids and hydroxy benzoate derivatives in lipid formulations, the solubilities in each lipid excipient were determined. The drug was added in excess to lipid excipient, vortexed for 4 minutes and then stored for 48 hours (for equilibration) at room temperature with shaking. Mixtures were then centrifuged at 2000g for 25 min using a Beckman centrifuge before a sample of the supernatant was taken, diluted with a suitable solvent, usually methanol 96%w/w for (hydrocortisone, testosterone and progesterone); ethanol (96% w/w) for (testosterone acetate and hydrocortisone acetate) and acetonitrile (99%w/w) for hydroxy benzoate derivatives; to be analysed by UV spectrophotometry. Cremophor RH 40® is solid material at room temperature; therefore, it was melted at 30°C before starting the experiment. The drug added to the melted surfactant and the mixture kept at 30°C for analysis.

2.3.3.2 Solubility of Steroids and hydroxy benzoate in lipid formulations

The solubility of each of the steroids and hydroxy benzoate derivatives were measured for each formulation and are shown in Table 2.3 using the above methods.

2.3.3.3 UV Assay

Quantitative determination of the concentration of steroid dissolved in the supernatant was achieved using an ultraviolet spectroscopic method. Each steroid and hydroxy benzoate derivative was analysed by scanning over a range of wavelengths (200 to

320 nm) using a double beam instrument (Perkin-Elmer Lambda7, UV / VIS Spectrophotometer). Standard curves for each of the drugs in the appropriate solvent were constructed and the peak wavelength determined. The Beer-Lambert law was closely obeyed for each drug (r^2 values of 0.99 were typical). The solubility of each drug was calculated using Beer's Law:

$$E^{1\%}_{1cm} = A/C_{g\%}.B \qquad \text{eq 2.1}$$

A stands for the absorbance of the solution, $C_{g\%}$ is the concentration absorbing substance in g / ml, and B is the path length (constant=1).

For the assay of drugs dissolved in excipients or formulations, the absorbance was determined against a blank of formulation diluted appropriately.

2.3.4 Particle size analysis of dispersion formed by surfactant-free formulation using the Malvern Mastersizer

Emulsions formed by surfactant-free (S-F) formulations were prepared by adding 1 ml of formulation to 100 ml of distilled water in a 250 ml conical flask using a 1 ml Gilson pipette. After mixing in a water bath shaker at 50 r.p.m, emulsions were visually grouped into 1 of 4 systems; 1) *crude dispersion* {Co}– indicating a formulation which showed poor or minimal emulsification with large oil droplets present on the surface; 2) *coarse emulsion* {Cr}– indicating a dull greyish white emulsion possibly with a slightly oily appearance; 3) *self-emulsifying system {S}*– indicating a bright white, milk like, emulsion; 4) *microemulsion* {M}– indicating a rapidly forming clear or slightly hazy bluish emulsion. The particle size analysis of S-F formulations was determined using an optical configuration represented by the Malvern Mastersizer presentation model 0807. Conventional Fourier optics were used with 100 nm and 300 nm lenses for particle sizes in the range of 0.3 to 600 μm. The emulsion was transferred from a conical flask to the mastersizer cell by means of

a glass pipette. Each dilution was performed in triplicate and the size distributions of the resultant emulsions compared using the apparent normal distribution of the diameter at 50 and 90 %, D (υ, 0.5) and D (υ, 0.9) respectively (Charman *et al*, 1992; Shah *et al*, 1994).

2.4 Results and discussion

2.4.1 Phase diagrams

Several SEDDSs formulations containing surfactants have been described previously (Constantinides *et al*, 1995; 1996). It was decided to investigate the formulation of a new type of 'Surfactant-Free' formulations, assessed drug solubility and investigate whether these formulations self–emulsify.

The ternary phase diagrams of systems containing oils with medium chain fatty acids, mixtures of mono-, di-, and tri-glycerides and water-soluble co-solvent, were studied and used to identify areas of mutual solubility and are shown in Figures 2.1–2.5. Miscibility depended on the nature of the oils, the hydrophilic co-solvent and their mixing ratios. Figures 2.1 and 2.2 showed phase diagrams of mixtures of mono-, di-, and tri-glycerides (Imwitor 988®), medium chain fatty acids (Miglyol 812®) and hydrophilic co-solvent PG and PEG 400 respectively. The phase diagrams showed that these components were able to form miscible areas as long as they contained at least 40% of mixture of glycerides, beyond which the systems become immiscible, and also a maximum 50% of medium chain glycerides. The PEG 400 systems (Figure 2.2) produced a much larger area of miscibility than those produced with PG (Figure 2.1). The larger miscibility region can be accounted for differences in the interaction of the medium chain fatty oil (Miglyol 812®) with the water-soluble co-solvents. The hydroxyl content of PG is also greater than PEG 400. Figure 2.3 represents that Transcutol P® produced large areas of miscibility in the presence of

MCT and mixtures of mono-, di-, and glycerides due to its lower hydrogen bonding capacity, whereas with glycerine there were no miscible mixtures.

To accommodate glycerine, four component mixtures were prepared, the hydrophilic component containing Glycerine/ PG (1:1) and/or Glycerine/ Transcutol P® (1:1). These are shown in Figures 2.4 and 2.5, respectively. This approach produced a miscible area provided at least 60%-70% of the mixture included glycerides (Imwitor 988®) and the formulation contained <30% medium chain oil (Miglyol 812®). As can be seen, the area of mutual solubility produced by the S-F systems is extended over a wide range of compositions controlled by both the polarity of the co-solvent and the mono-, di-, and tri-glyceride content of the oil. Long chain triglycerides produce very few miscible S-F formulations even with PEG 400 or Transcutol P®. This is because the co-solvents are too hydrophilic to mix with the hydrophobic long chain triglycerides.

It is clear from the phase diagrams that the S-F formulations are clear, transparent, isotropic and thermodynamically stable. They are typically isotropic mixtures of medium chain oils <40% (Miglyol 812®), mixtures of mono-, di-, and tri-glycerides >40% (Imwitor 988®) and water soluble co-solvent <30% (Transcutol P®, PG, PEG 400). Since the formation of the S-F formulation is thought to be thermodynamically stable, the order of the addition of the components should not have any effect on the formulations.

2.4.2 Drug incorporation into lipid excipients of SEDDSs formulations

In properly selecting a suitable lipid system for drug solubilization and delivery, it would be useful to have pre-formulation data particularly on aqueous solubility, and/or lipid excipients formulations or binary mixtures of lipid excipients. This should allow the formulator to make an early choice between the types of SEDDSs

formulations and to predict for which drugs the solvent capacity can be improved by the use of co-solvents (Constantinides, 1995).

Steroids and hydroxy benzoate derivatives were used as models of BCS class II drugs, being poorly water-soluble, highly membrane permeable drugs. Solubility studies were carried out for steroids in lipid excipients and SEDDS formulations. Drugs were assayed by UV spectrophotometry. Each experiment was repeated under the same conditions three times. Statistical analysis was not undertaken for each experiment due to complexity of the systems studied.

The steroids were selected as poorly water-soluble drugs, with log *P* (1-4). Their oil solubility was limited. Hydroxy benzoate derivatives with log *P* (1-4) were used with high oil solubility. Therefore, control experiments were done to ensure that the equilibrium solubility is estimated accurately.

2.4.2.1 Determination of the equilibrium solubility of steroids

Formulations of SEDDSs and their individual excipients are of a viscous nature; therefore, it was not easy to predict the length of time required to reach equilibrium. It was convenient to use gentle heating to speed dissolution, but it was important to make sure that the solubility measurements after re-equilibration at room temperature were true values rather than measurements of the supersaturated state.

Increasing the temperature resulted in a decrease in the surface tension of the solvent and a decrease in the viscosity of the formula, thus increasing the dissolution rate and solubility of the solute in the formula. The solubility of steroids was studied in surfactant-free formulations by either shaking at 25°C or mixing at 50°C followed by re-equilibration at 25°C for different time intervals. Figures 2.6-2.9 show the effect of pre-heating on the apparent solubility of steroids at 25°C in S-F formulations. At the first time point, there was 2-fold more drug in solution for samples pre-heated at

50°C than at 25°C. At 25°C, the solubility of steroids exhibits nearly the same solubility at 50°C after approximately 96 hours. Figure 2.6 showed that progesterone exhibited 2.744 g % w/v ± 0.7 %, after 96 hours at 25°C, and at 50°C 2.298 g % w/v ± 0.6 %. Figure 2.7 indicated that testosterone required 96 hours to reach equilibrium. The amount of testosterone solubility at 25°C was 2.603 g % w/v, ± 0.3% was very close to the amount dissolved in the pre-heated samples 2.815 g% w/v, ± 0.4%. The solubility of testosterone acetate at 25°C after 96 hours was 2.07g%w/v ± 0.4% whereas at 50°C after 96 hours was 2.394 g %w/v ± 4%. Figure 2.8 showed that at 25°C, the solubility of hydrocortisone 21-acetate slightly increased up to 12 hours then decreased until it reached equilibrium after 192 hours.

Figure 2.9 showed that increased sharply the initial solubility of pre-heated testosterone samples, which indicated that the drug reached a supersaturated state when pre- heating was used. The samples took at least 96 hours to reach equilibrium. Nearly all the pre-heated samples exhibited the same % w/v solubility 3.0 % w/v, ± 0.4 % at the end of four days.

Therefore, the protocol used for determining a single point of solubility was to mix lipid excipients in the presence of the drug for 2–3 minutes then left for equilibration for at least 48-96 hours before further analysis was undertaken.

2.4.2.2 Determination of the intrinsic solubility of steroids and hydroxy benzoate derivatives in lipid excipients.

The partition coefficient of the drug (Log *P* octanol/water) is an important indicator of the likely solubility properties of a drug. For example, the solubility of hydrocortisone with a log *P* value of 1.53 in lipid excipients can be ranked for their solvent capacity: Imwitor 988® (1.55 g % w/v) > Capmul MCM® (1.002 g % w/v) > Cremophor RH40® (0.733 g % w/v) > Miglyol 812® (0.1 g % w/v) ≈ Tagat To®

(0.1 g % w/v). However, for hydrocortisone acetate (Log *P* 2.7) the rank was Miglyol 812® (0.386 g % w/v) > Imwitor 988® (0.22 g % w/v) > Cremophor RH 40® (0.147 g% w/v) > Capmul MCM® (0.1224 g% w/v) > Tagat To® (0.025 g % w/v). Both hydrocortisone and the acetate form produce a significant solvent capacity in the hydrophilic co-solvent. For example, the intrinsic solubility of hydrocortisone can be ranked in the following order: PEG 400 (1.228 g % w/v) > Transcutol P® (1.077 g % w/v) > PG (1.0 g % w/v). The acetate form can be ranked in the following manner: Transcutol P® (0.78 g % w/v) > PG (0.367 g % w/v) > PEG 400 (0.333 g % w/v). Therefore, the intrinsic solubility of hydrocortisone and the acetate form would be highly sensitive to the formulation contents.

As the partition coefficient increased as in case of testosterone (Log *P* 3.3) the rank was: Cremophor RH 40® (0.975 g % w/v)> Miglyol 812 ® (0.847 g % w/v) Imwitor 988® (0.816 g % w/v) >Capmul MCM® (0.79 g % w/v)> Tagat To® (0.301g % w/v). While, the acetate form (Log *P* 4.7) the rank was: Tagat TO® (1.274 g % w/v) > Imwitor 988® (1.10 g % w/v) > Cremophor RH 40® (1.60 g % w/v) > Capmul MCM® (1.04 g % w/v) Miglyol 812® (1.035 g % w/v). Both testosterone and the acetate form are exhibited better solvent capacity in hydrophilic co-solvents. Testosterone was ranked in the following manner: Transcutol P® (1.11 g % w/v) > PEG 400 (0.95 g % w/v) > PG (0.822 g % w/v).

Testosterone acetate, also, exhibited the same order of the solvent capacity in the hydrophilic co-solvent: Transcutol P® (1.142 g % w/v) > PEG 400 (0.92 g % w/v) > PG (0.87 g % w/v). Therefore, testosterone may produce better solvent capacity in a mixture of hydrophilic surfactant (HLB >12) mixed with mono-, di-, and tri-glycerides in the presence of hydrophilic co-solvent *i.e.* Type III. On the other hand, the acetate form may produce better solvent capacity in a mixture of hydrophilic surfactant (HLB<12) and mixed mono-, di and tri- glycerides (Imwitor 988® and Capmul MCM®) *i.e.* Type II. Progesterone (log *P* 3.8) exhibited the following rank: Imwitor 988® (1.6 g % w/v) > Cremophor RH 40® (1.21 g % w/v) Capmul MCM®

(1.2 g % w/v) > Miglyol 812® (1.147 g % w/v) > Tagat TO® (0.83 g % w/v). Therefore, progesterone intrinsic solubility could be much better in mixed glycerides, mono-, di-, and tri-glycerides with or without hydrophilic surfactant (HLB > 12) in the presence or absence of hydrophilic co-solvents.

However, in the case of hydroxy benzoate derivatives all the derivatives exhibited a much higher g % w/v solubility in Imwitor 988® (9.0 g % w/v - 27.0 g % w/v), Miglyol 812® (7.0 g % w/v-12 g % w/v) compared with the steroid derivatives (0.2 g % w/v-1.5 g % w/v and 0.03 g % w/v-1.1 g % w/v) respectively. This is most likely to the molecular volume; the parabens have lower molecular weight than the steroids and their lower melting points which indicates lower crystal lattice energy.

In general, the co-solvent can be a good solvent for hydrophobic drugs when the polarity is intermediate, as seen with Transcutol P®. For example, hydrophobic drugs like hydrocortisone acetate, testosterone, and testosterone acetate was observed a higher solubility in Transcutol P® 0.779 g %w/v, 1.11 g % w/v and 1.14 g %w/v respectively.

Mixed glycerides, mono-, di-, and tri- glycerides (Imwitor 988® or Capmul MCM®) could be very much better solvent than in lipid formulations for most of hydrophobic drugs.

2.4.2.3 Determination of the intrinsic solubility of steroids and hydroxy benzoate derivatives in SEDDS formulations

The solubility of hydrophobic drugs in lipid excipients gives an early indication of the solubility in the SEDDSs formulations. The solubility of steroids in an S-F formulation was greater than in the lipid excipients and/or SEDDS formulations. The greatest solubility was measured in a S-F formulation with PG as a water-soluble co-solvent. In the case of testosterone, the solubility was 2.568 %w/v and the acetate

form was 2.06 g%w/v. Hydrocortisone exhibited the highest solubility in the presence of PG in surfactant- free formulations 1.203 g%w/v. While progesterone and the hydrocortisone acetate exhibited better solvent capacity in the presence of Transcutol P® in the S–F formulations 2.598 g%w/v and 0.178 g%w/v, respectively.

Steroids with a high log P>3 showed the highest solvent capacity in mixtures of mono-, di-, and tri-glycerides, hydrophilic co-solvents and medium chain triglycerides oil. On the other hand, the solvent of mixture of mono-, di- and tri-glycerides could be the best solvent for steroids with low log P<3.

Pouton (1999) classification of SEDDS formulations into three categories based on their hydrophilic content is shown in Table 2.4. The solubility of steroids studied in the SEDDS formulations was compared to his classification system.

Figures 2.10-2.12 show the effect of different ratios of Miglyol 812®: Tagat To® on solubility. From the data showed, type II formulations are drug dependent. For example, Figure 2.10 indicated that as the percentage of TTO® increased from 20 % to 80%, the solubility of hydrocortisone increased from 0.105 g % w/v to 0.418 g % w/v. However, the acetate form showed a better solubility when the percentage of Miglyol 812® was increased from 20% to 80% in the formulation, 0.01 g % w/v to 0.069 g % w/v respectively. This result was expected because the solubility of hydrocortisone acetate in the M 812® was 0.386 g%w/v higher than in TTO® 0.025 g % w/v. Although, the value was lower than expected. This means that the drug is more sensitive to the formulation and a type I (Miglyol 812®) could be the best solvent. Figure 2.11 showed that testosterone and its acetate form followed the same order of solubility as hydrocortisone acetate. As the percentage of medium chain triglycerides oil increased from 20% to 80%, the solubility of testosterone and its acetate form increased from 0.504 g % w/v to 0.82 g % w/v and from 0.459 g % w/v to 0.69 g % w/v respectively. Both drugs exhibited better solvent capacity in the presence of medium chain triglycerides (Miglyol 812®) than mixed with a hydrophilic

surfactant. The solubility of testosterone in M 812® was 0.874 g % w/v and the acetate form was 1.035 g % w/v respectively. Figure 2.12 illustrated that progesterone followed the order of hydrocortisone, as the percentage of TTO® increased from 20 % to 80 % the solubility increased from 0.81 g % w/v to 1.507 g % w/v. Type II formulations have the advantage that they are less likely than type III formulations to lose their solvent capacity after emulsification, but will have lower solvent capacities than type III systems for drugs with intermediate log *P* and high molecular weight as for steroids.

Moving from Type II system to Type III, the solubility was enhanced due to an increase the hydrophilic content. In Type III A, the experiment indicated that the solubility of hydrocortisone and its acetate ester would be at a maximum of 0.77 g % w/v and 0.206 g % w/v respectively when the concentration of the surfactant and water-soluble co-solvent was used at a ratio of 1:1. Progesterone, testosterone and its acetate ester exhibited maximum solubility, 1.364 g % w/v, 1.034 g % w/v and 1.104 g % w/v respectively when the ratio of surfactant to water-soluble co-solvent was 3:2.

As the hydrophilic content (Cremophor RH40®, 40 %) was increased the solubility of steroids increased as shown in type III B. In general, type III are suitable for drugs with an intermediate Log *P* (1-4) as in the case of steroids and hydroxy benzoate derivatives. Hydroxy benzoate derivatives exhibited a higher % w/v solubility in SEDDS formulations compared to the steroids. For the hydroxy benzoate derivatives, the more hydrophilic the drug, the more soluble in high-content hydrophilic SEDDS formulation *e.g.* methyl paraben log *P* (1.6) indicated higher % w/v solubility 50.0 g % w/v 7 4 % in type III B. Typically, hydroxy benzoate derivatives (log *P* >2) are soluble in the following order: S-F>Type III B > Type III A > Type II, related to their solvent capacity in the SEDDs formulation.

Further studies were done to investigate whether a linear relationship exists between the content of glycerides and the solubility of the steroids. These are shown in Figures 2.13-2.14. In general, as the weight fraction of mono-, di-, and tri-glycerides (Imwitor 988®) increased the % w/v solubility increased. It can be seen in Figure 2.13 that as the weight fraction of glycerides to medium chain oil increased the solubility of hydrocortisone and acetate ester increased. The solubility of mixtures went hand-in-hand with the solubility of the excipients; *e.g.* the % w/v solubility of hydrocortisone acetate in ratio (3:7) Miglyol 812®: Imwitor 988® was 0.117 g % w/v, whereas the intrinsic solubility in Imwitor 988® was 0.22g % w/v. The same order can be applied in Figure 2.14, where the solubility of testosterone and its acetate ester increased when the ratio of Imwitor 988®: Miglyol 812® increased 7:3.

Figures 2.15-2.17 showed that water-soluble binary mixture of mono-, di-, and tri-glycerides and hydrophilic co-solvent (Imwitor 988® with PG and/or PEG 400) were tested for their solvent capacity using steroids. All the steroids, exhibited greater solvent capacity (% w/v) in these binaries than in SEDDS formulations. The type of water-soluble co-solvent affects the solvent capacity. The optimum ratio of mixtures of mono-, di and tri-glycerides and hydrophilic co-solvent is drug dependent. Figure 2.15 showed that progesterone exhibited the maximum solubility at ratio (3:7) Imwitor 988®:PG and/or PEG 40. The solvent capacity was 1.68 g % w/v and 1.90 g % w/v, respectively. The same order can be seen in Figure 2.16, where hydrocortisone exhibited the maximum solvent capacity at a ratio 3:7 Imwitor 988®: PG and/or PEG 400. The solubility was 1.871 g % w/v and 1.631 g%w/v, respectively. Whereas hydrocortisone acetate exhibited the maximum solvent capacity at a ratio of 5:5 where the solvent capacity was 0.251 g % w/v. Figure 2.17 showed that in the presence of PEG 400 as hydrophilic co-solvent the maximum solvent capacity of testosterone was at ratio of 3:7 Imwitor 988®: PEG 400 0.897 g % w/v. However, in the presence of PG as a hydrophilic co-solvent the maximum solvent capacity was at a ratio 5:5, 2.39 g%w/v. Testosterone acetate exhibited that maximum solvent capacity in the presence of PEG 400 at a ratio 3:7 Imwitor 988®:

PEG 400, 1.19 g%w/v and in the presence of PG the ratio was 8:2 Imwitor 988®: PG, 0.965 g%w/v. In general, binary mixtures of PEG 400 and Imwitor 988® produced better solvent capacity for all of steroids except for testosterone.

2.4.3 Particle size analysis of surfactant-free formulations

Surfactant-free formulations, which exhibited miscibility are indicated in the phase diagrams Figures 2.1 – 2.5. The characterization of the dispersion were performed using the water dispersion model. From inspection of the phase diagrams, most of the formulations exhibit crude to coarse emulsions upon dispersion into water. The effect of co-solvent concentration, in mixtures of glycerides, on droplet size distribution is presented in Figures 2.18 and 2.19. Figure 2.18 represented that S-F formulation containing mixtures of mono-, di- and tri-glycerides <50%, and increasing the co-solvent concentration (from 10 % to 30 %) decreased the mean droplet size. A smaller droplet size was observed when the co-solvent concentration was in the range <15% Transcutol P® and the co-surfactant was in the range 50-90 % mixture of glycerides. Such decrease in droplet size may reflect the formation of a better close packed film of the cosurfactant at the oil-water interface, thereby stabilizing the oil droplet (Kommuru *et al*, 2001).

The type of co-solvent affects particle size, as shown in Figure 2.19. This indicated the effect of increasing the concentration of different co-solvent on a fixed ratio of Imwitor 988®: Miglyol 812® (6:4). Transcutol P® indicates the smallest particle size among the other hydrophilic co-solvents. Although, the addition of co-surfactant (Imwitor 988®) to the co-solvent lowered the interfacial tension, fluidised the hydrocarbon region of the interfacial region of the interfacial film, and decreased the bending stress of the interface, the decrease in droplet size was not significant. Kommuru *et al* (2001) has reported that the presence of co-solvent in microemulsion formulation increased the droplet size.

2.5 Summary of results and conclusions

The mixed glyceride (mono-, di-, tri-glycerides) excipients, Imwitor 988® and Capmul MCM®, were particularly good solvents for all of the corticosteroids particularly steroids with log P<2. There was a marked difference in solubility of steroids in MCT oil and, consequently, it was considered that the addition of co-solvent might improve the solvent capacity of triglyceride-based systems. Surfactant-free formulation are typically composed of mixtures of Transcutol P® < 30 %, Imwitor 988® >50 % and Miglyol 812® >30 %. Surfactant-free (type IV) formulations were generally better solvents than SEDDS type II formulations but were generally not superior solvents to mixed glycerides of mono-, di-, and tri-glycerides (Imwitor 988 ®) and (Capmul MCM®) alone. Type III SEDDS, which are hydrophilic, were also better solvents for most of the steroidal compounds than type II SEDDS. Although the homologous series of steroids span a considerable range of log P, their solubilities across a range of excipients and formulations were surprisingly similar, generally ranging from 0.2-2.1 g % w/v. Hydrocortisone acetate had a lower solubility in most systems than the other steroids, and generally, progesterone showed the highest solubility. On the contrary, for hydroxy benzoate derivatives, as the log P increased the solubility decreased, in most systems.

Figure 2.1 Phase diagram of mono-, di-, and tri- glycerides, medium chain fatty acids oil and propylene glycol as water-soluble co-solvent

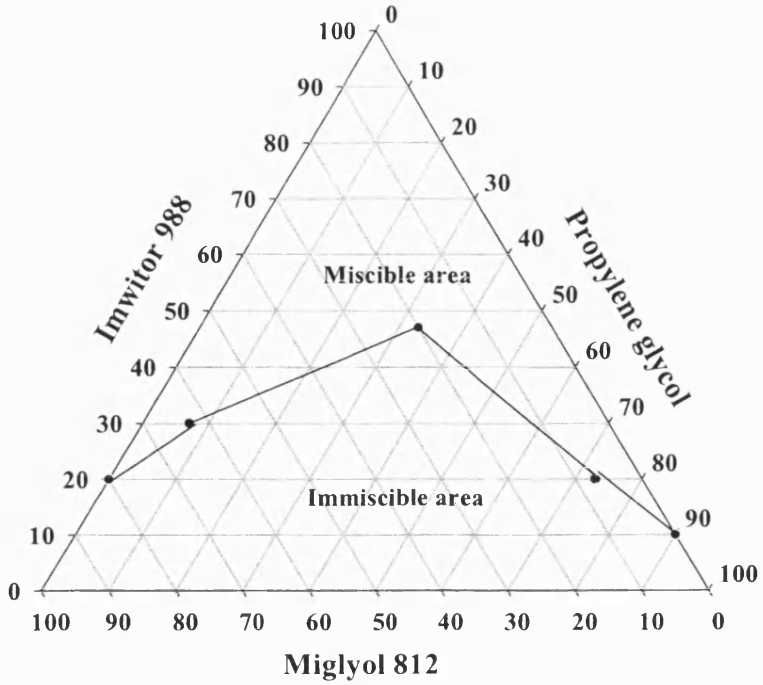


Figure 2.2 Phase diagram of mono-, di-, and tri-glycerides and medium chain fatty acids oil and polyethylene glycol (PEG 400) as hydrophilic co-solvent

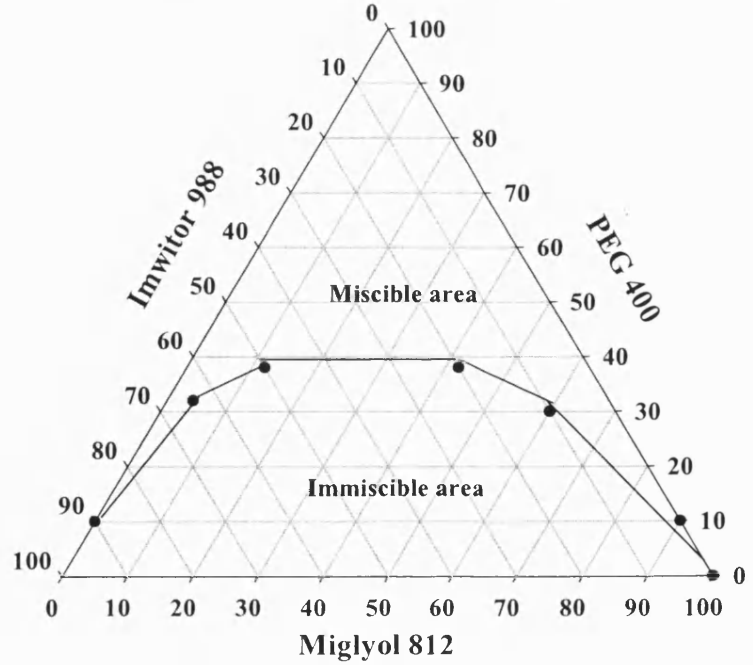


Figure 2.3 Phase diagram of mixture of mono-, di-, and tri-glycerides and medium chain oil, and Transcutol P as hydrophilic co-solvent

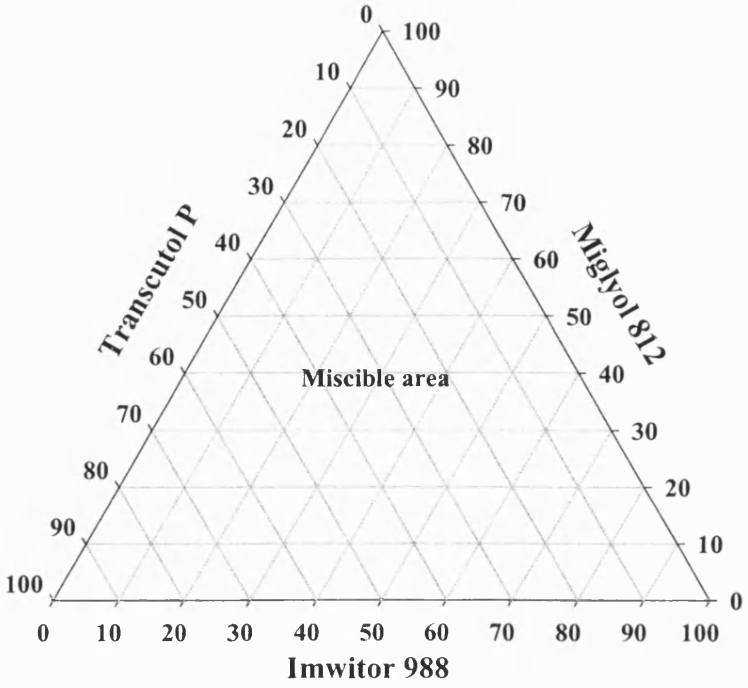


Figure 2.4 Phase diagram of mixture of mono-, di-, and tri-glycerides, medium chain oil and mixture of hydrophilic co-solvent

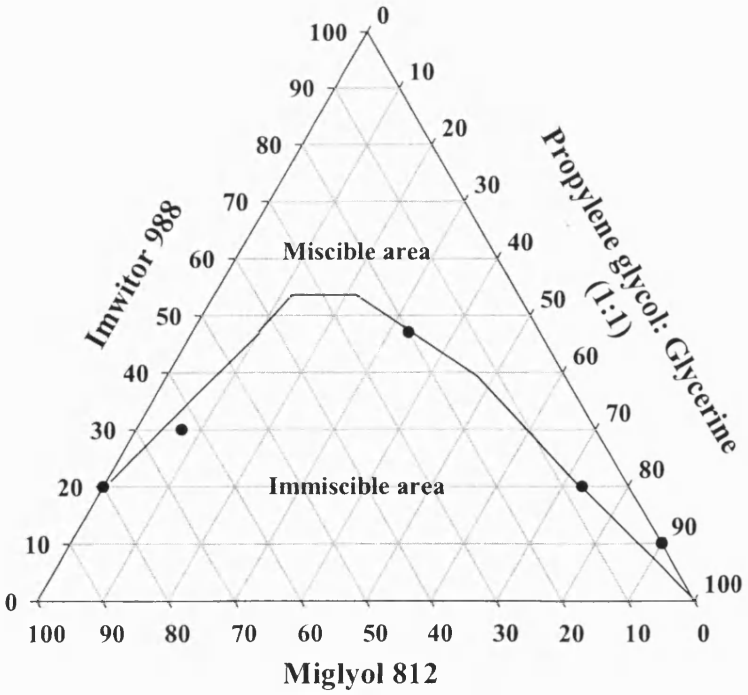


Figure 2.5 Phase diagram of mixture of mono-, di-, and tri- glycerides and medium chain oil and mixture of hydrophilic co-solvents

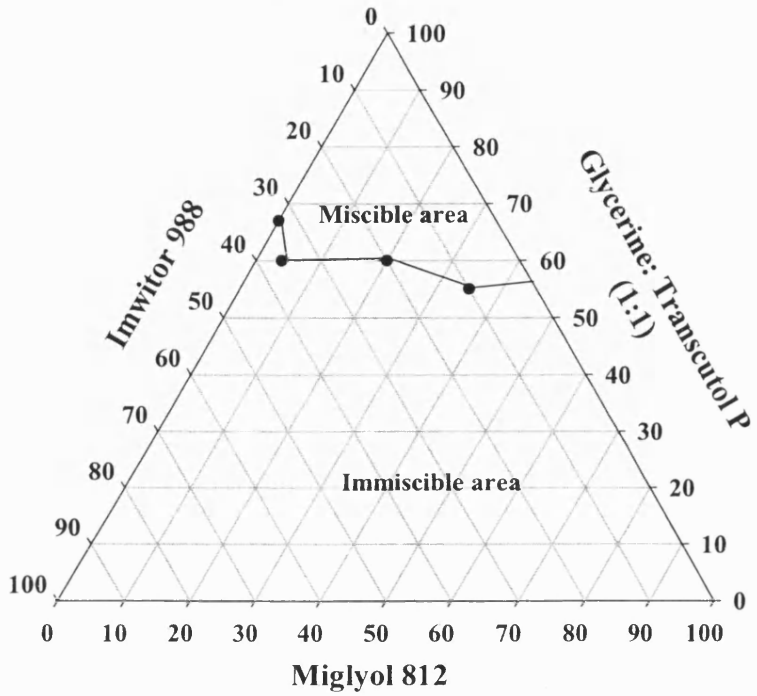


Figure 2.6 Solubility profile of progesterone in surfactant-free formulations at 25°C and 50°C, respectively at different time intervals (the error bars represent the standard deviation of three experiments)

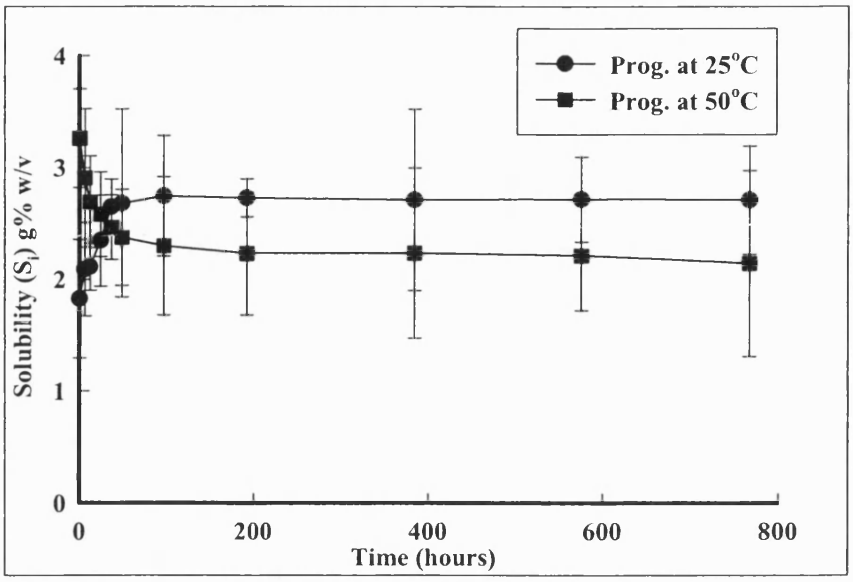


Figure 2.7 Solubility profile of testosterone and its acetate ester in surfactant-free formulations at 25°C and 50°C, respectively at different time intervals (the error bars represent the standard deviation of three experiments)

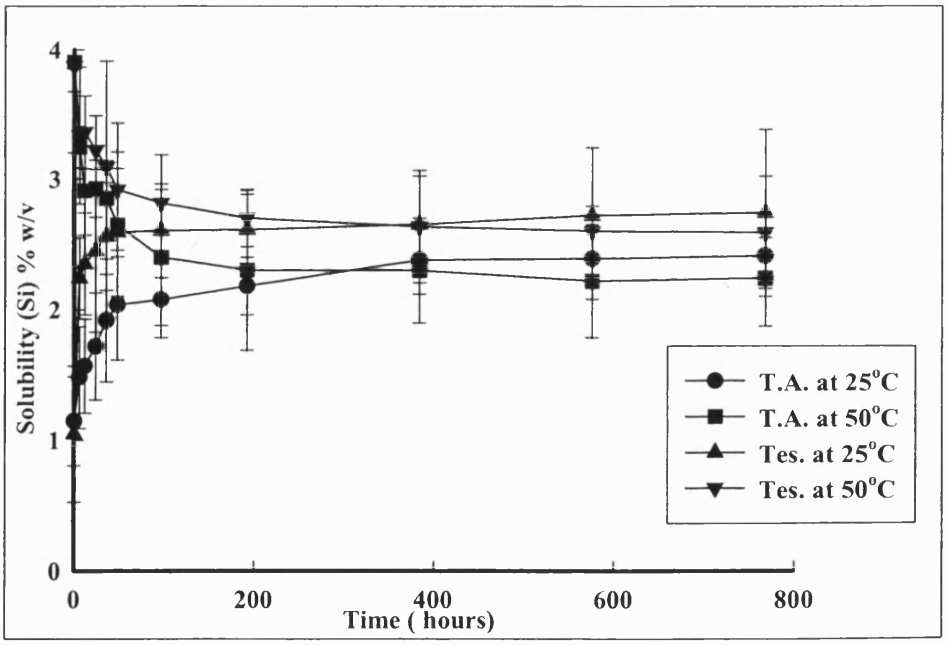


Figure 2.8 Solubility profile of hydrocortisone and its acetate form in surfactant-free formulations at 25°C and 50°C, respectively at different time intervals(the error bars represent the standard deviation of three experiment)

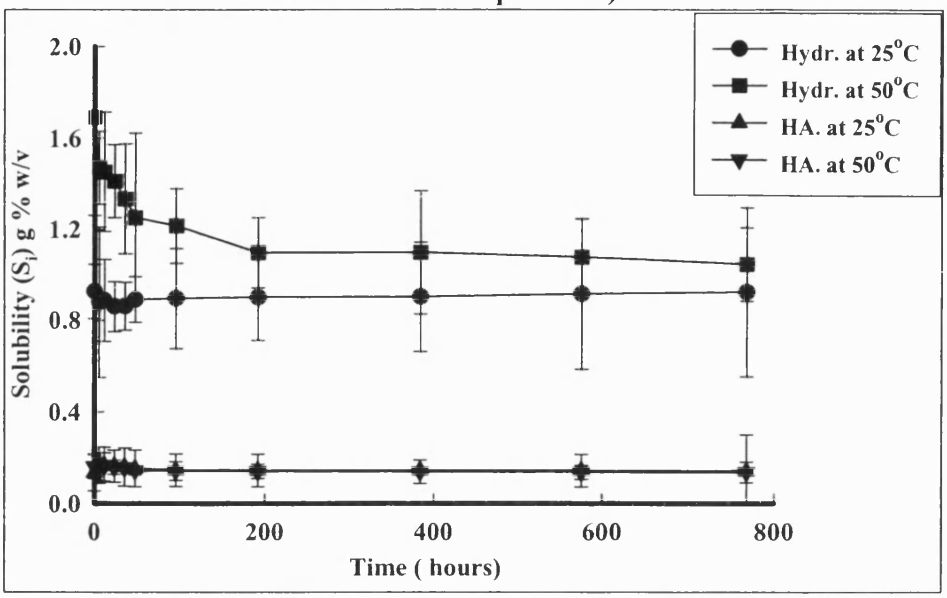


Figure 2.9 Solubility profile of testosterone in surfactant-free formulations at different temperatures vs time intervals(the error bars represent the standard deviation of three exerpiments)

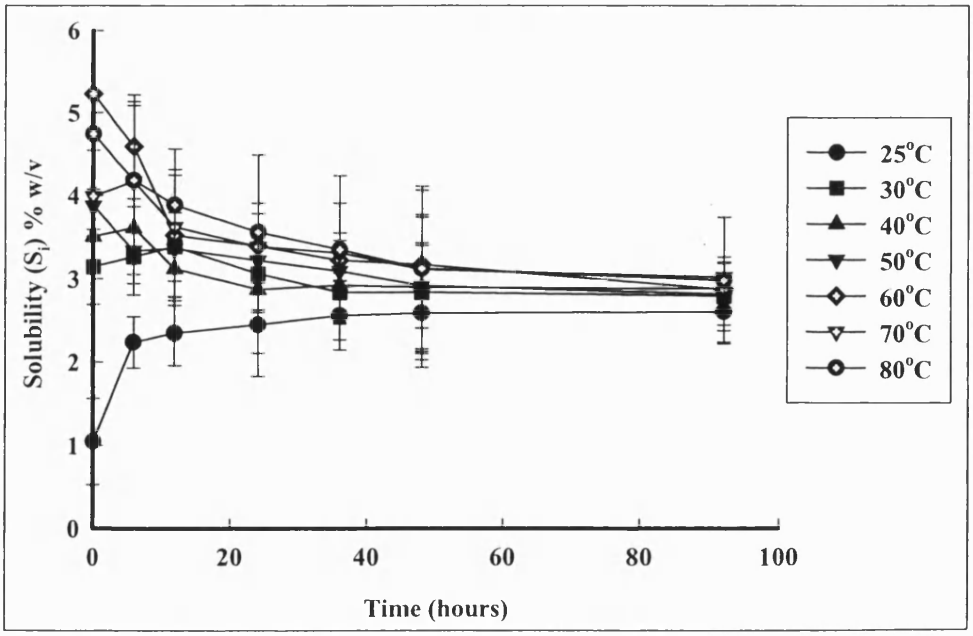


Figure 2.10 Solubility profile of hydrocortisone and its acetate form in different ratios of type II formulations of MCT and glyceryl trioleate surfactant (Miglyol 812: Tagato TO) at 25°C (the error bars reprsent the standard deviation of three experiments)

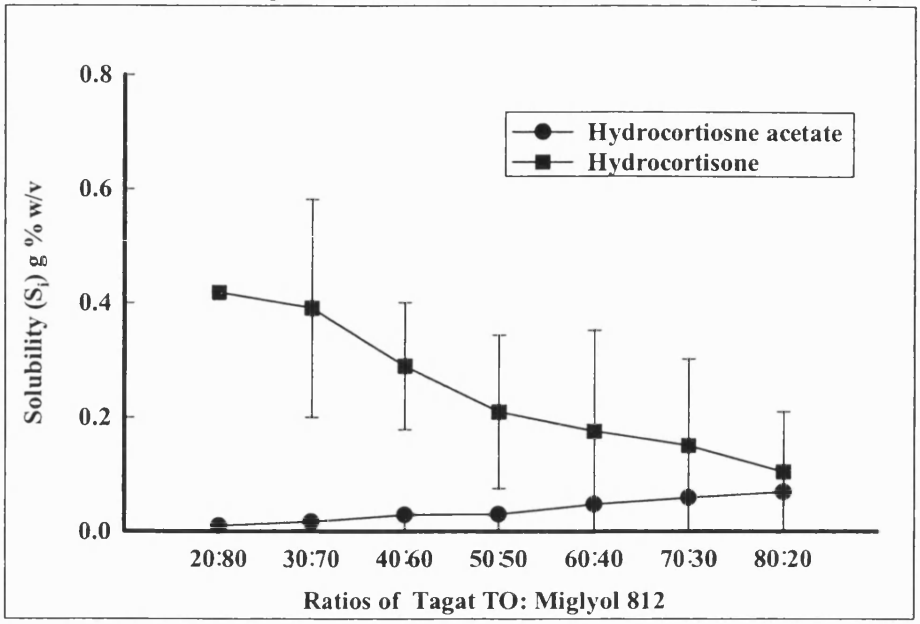


Figure 2.11 Solubility profile of testosterone and its acetate form in different ratios of type II formulations of MCT and glyceryl trioleate surfactant (Miglyol 812: Tagat TO) at 25°C (the error bars reprsent the standard deviation of three experiments)

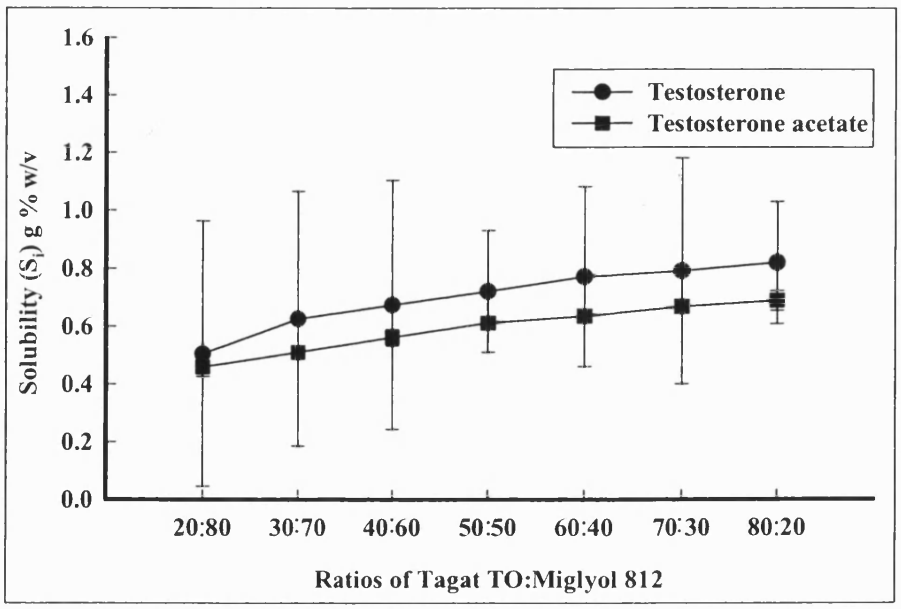


Figure 2.12 Solubility profile of progesterone in type II formulations in different ratios of MCT and glyceryl trioleate (Miglyol 812 : Tagat TO) and mixed mono-, di- and tri-glycerides and MCT (Imwitor 988 : Miglyol 812) at 25°C (the error bars represent the standard deviation of three experiments)

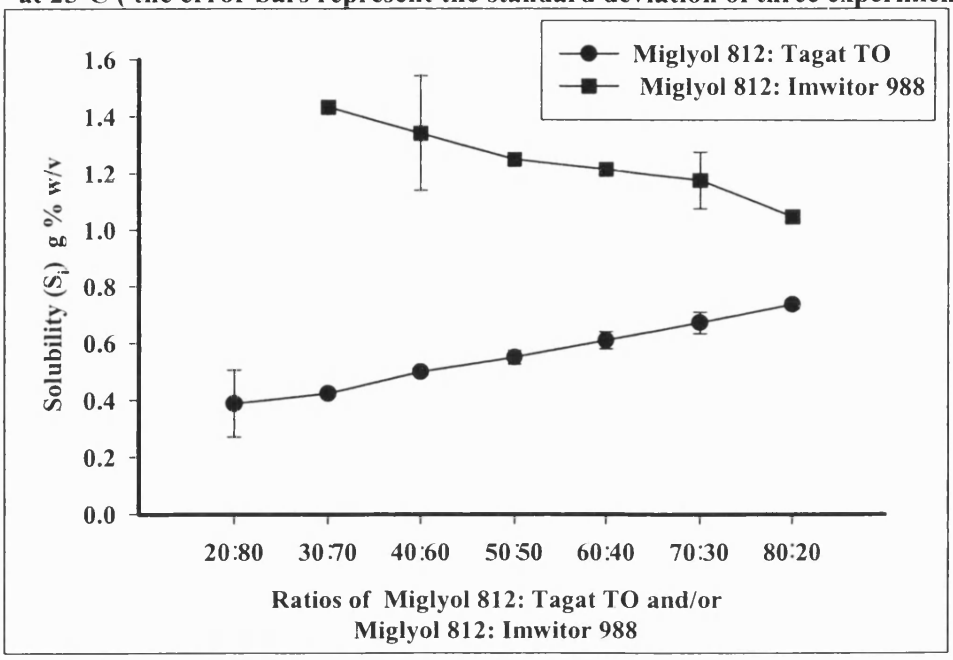


Figure 2.13 Solubility profile of hydrocortisone and its acetate form in mixture of mono-, di- and tri-glycerides and MCT(Imwitor 988 : Miglyol 812) at 25°C (the error bars reprsents the standard deviation of three experiments)

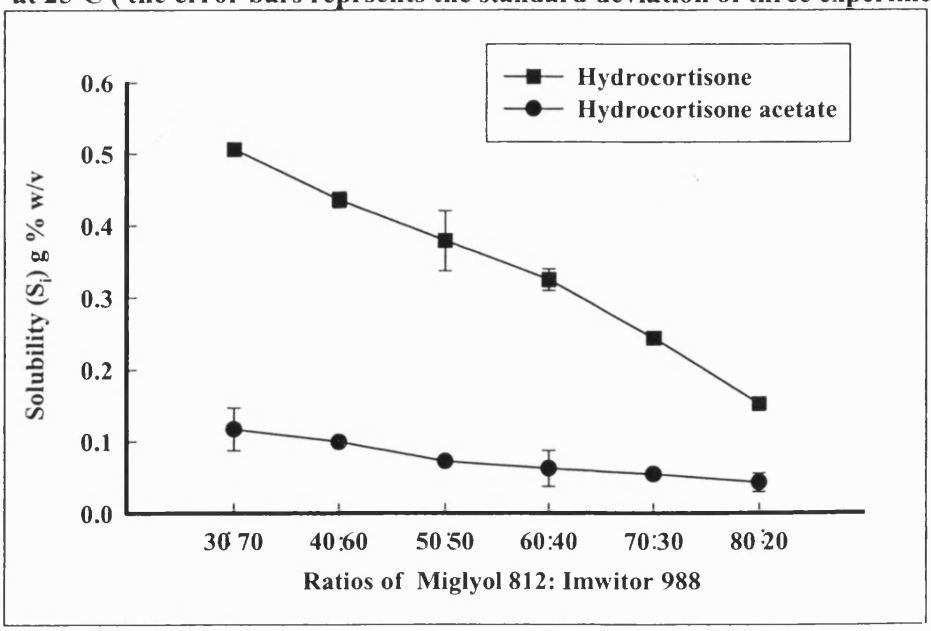


Figure 2.14 Solubility profile of testosterone and its acetate form in mixtures of mono-, di-, and tri-glycerides MCT (Imwitor 988 : Miglyol 812) at 25°C (the error bars reprsent the standard deviation of three experiments)

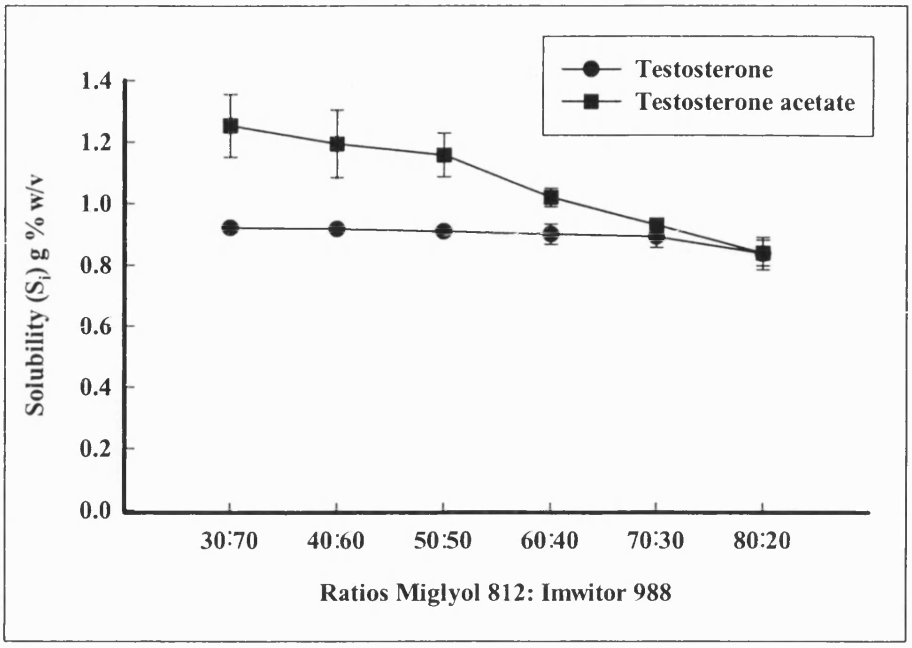


Figure 2.15 Solubility profile of progesterone in mixtures of mono-, di-, and tri-glycerides and hydrophilic co-solvents at 25°C (the error bars represent the standard deviation of the three experiments)

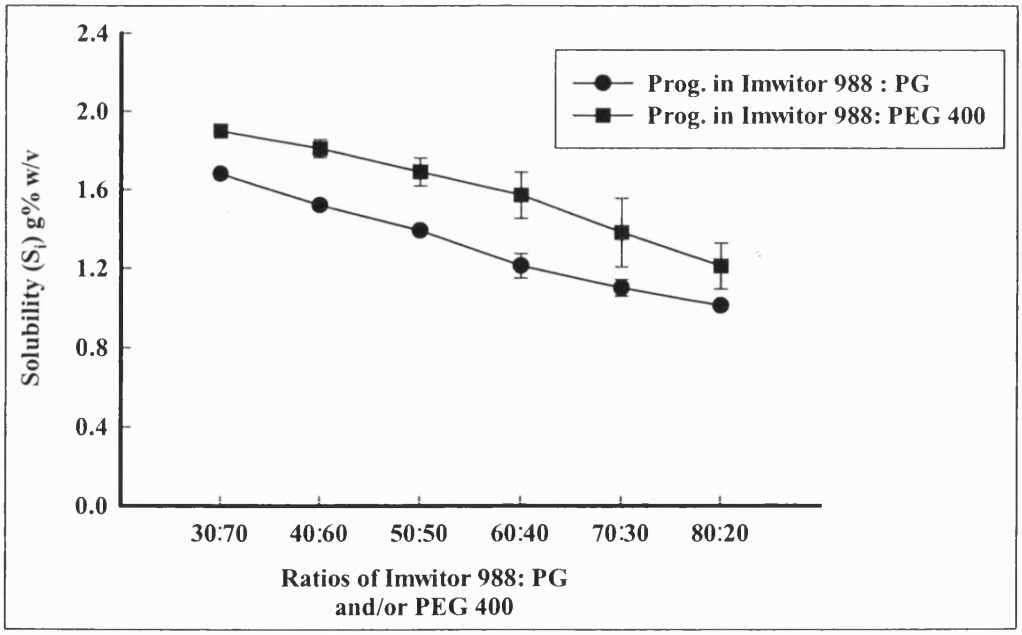


Figure 2.16 Solubility profile of hydrocortisone and its acetate form in mixtures of mono-, di-, and tri-glycerides and hydrophilic co-solvent at 25°C (the error bars reprsent the standard deviation of three experiment)

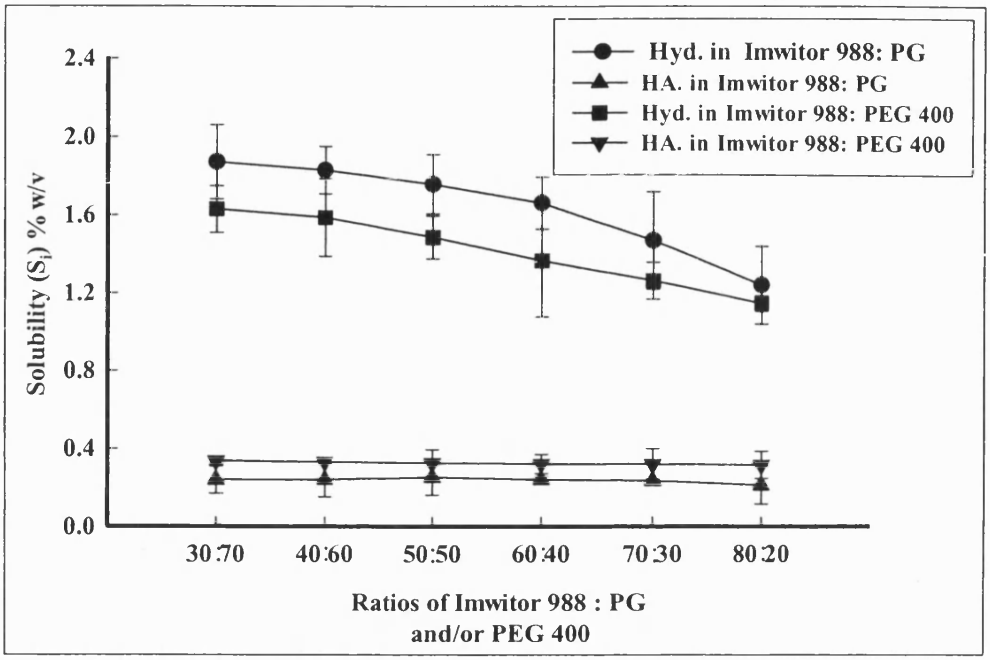


Figure 2.17 Solubility profile of testosterone and its acetate form in mixtures of mono-, di-, and tri-glycerides and hydrophilic co-solvent at 25°C (the error bars represent the standard deviation of three experiments)

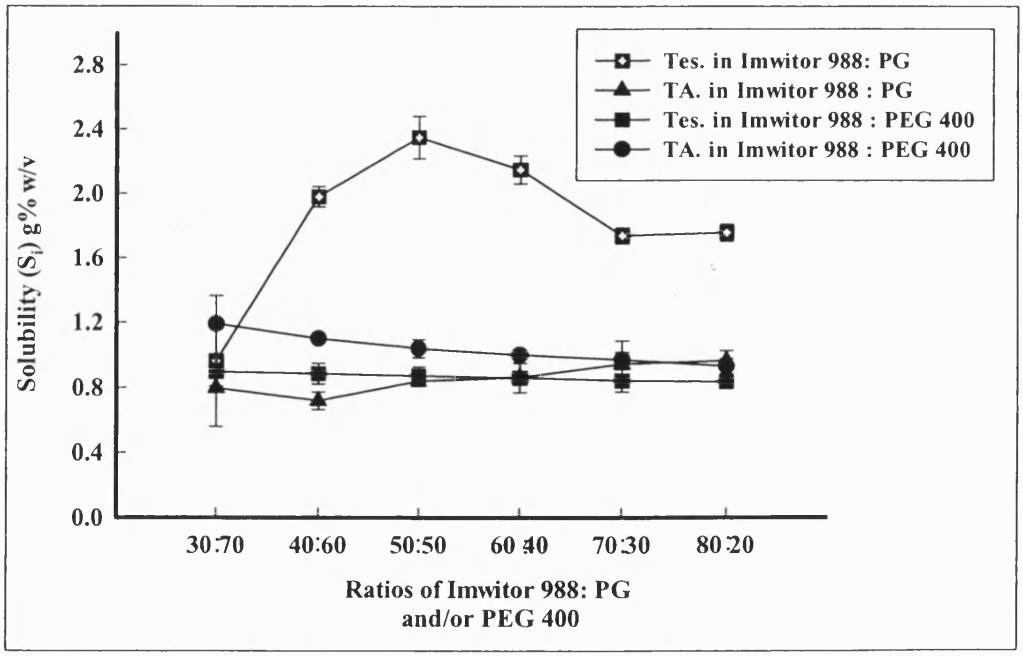


Figure 2.18 Effect of adding hydrophilic co-solvent (Transcutol P)on mixtures of mono-, di-, and tri-glycerides and medium chain fatty acids oil (Imwitor 988 : Miglyol 812) on the particle size distribution (the error bars represent standard deviation of three experiments).

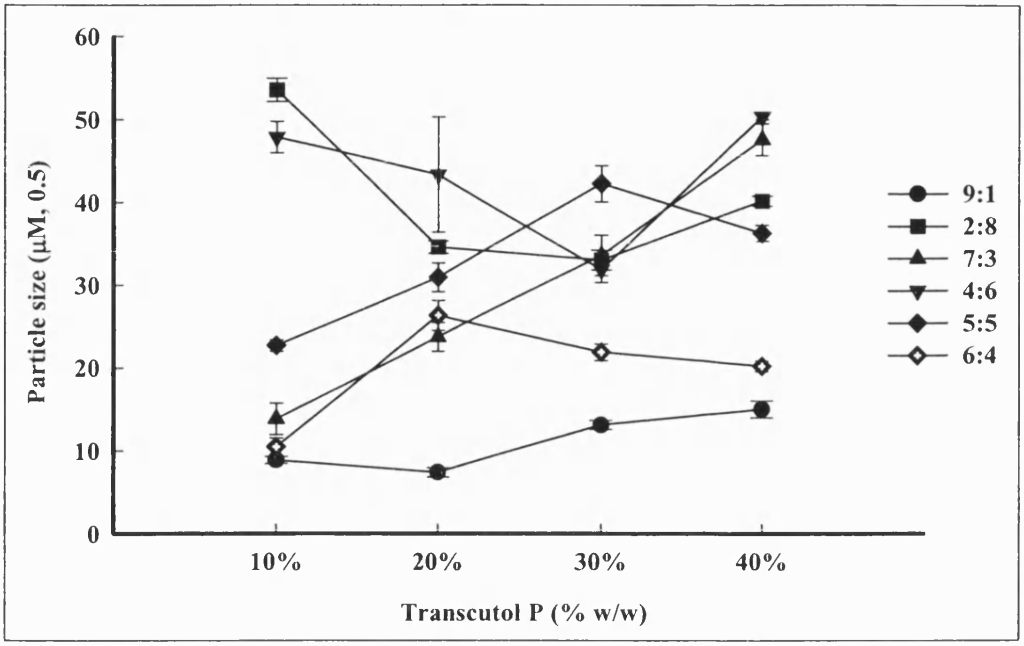
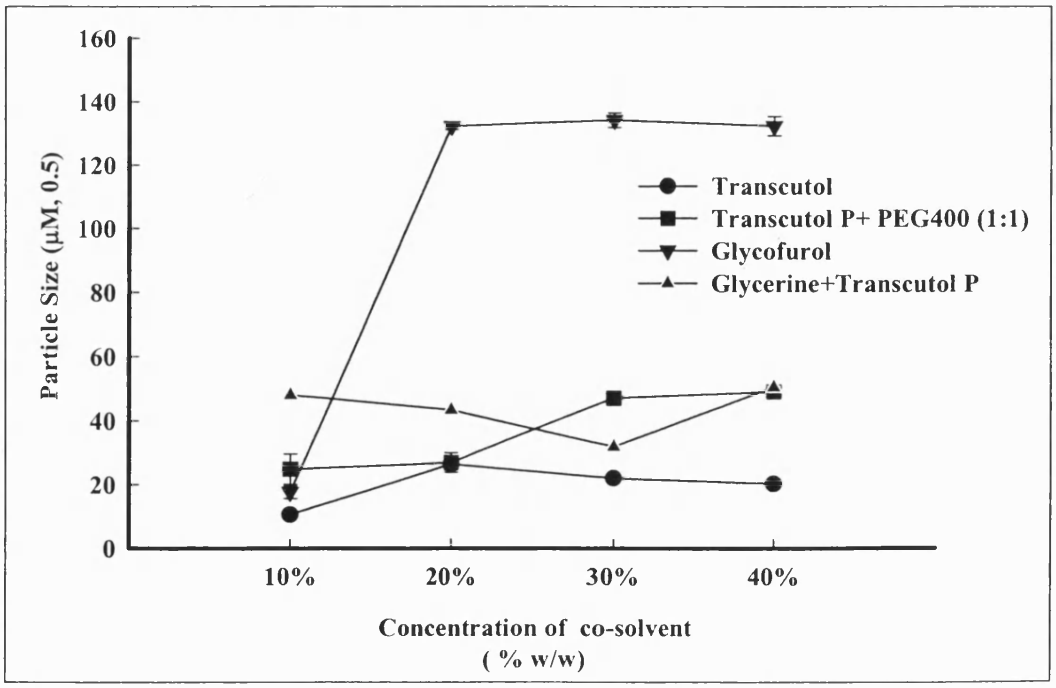


Figure 2.19 Effect of adding different hydrophilic co-solvent on a mixture of mono-, di-, and tri-glycerides and medium chain fatty acids oil (Imwitor 988 : Miglyol 812) (6:4) on particle size distribution (the error bars represent the standard deviation of three experiments)



Chapter 3

Assessing the fate of hydrophobic drugs after dispersion of lipid formulations in aqueous media

3.1 Introduction

Lipid systems for oral administration can include triglycerides, diglycerides, monoglycerides, lipophilic surfactants, hydrophilic surfactants, and water-miscible co-solvents. The fate of lipid formulations, and the drug, after oral administration will depend on the aqueous solubility of the individual components, and the phase changes that occurs on dilution into an aqueous medium. Phase changes and dissolution of water-miscible components could lead to loss of overall solvent capacity and precipitation of the drug. This is undesirable because the biopharmaceutical advantage of a lipid formulation is the possibility that the drug can remain in solution in the gut, thereby avoiding the slow dissolution step, which can cause low and variable bioavailability from solid dosage forms. Aqueous solubility plays an important role in the determination of a drug bioavailability (Morelock *et al*, 1994). Since a drug must be soluble in the gastrointestinal fluid to be orally active, the rate and extent of dissolution depend critically upon water solubility (Yalkowsky *et al*, 1980). Despite the potential of lipid formulation vehicles, very few studies to date have systematically examined the fate of the hydrophobic drugs in aqueous solution of lipid excipient and/or lipid formulation. The solubility of the drug is determined by the interaction of solute with solvents and the crystallinity of the solute (Ran *et al*, 2001). Solvent alteration is the most effective means to produce a thermodynamically stable increase in solubility. Yalkowsky *et al,* (1981) discussed the most commonly used approaches for solubilizing nonionizable drugs, such as steroids; namely cosolvency and micellization.

Co-solvency, the addition of water miscible solvents, such as ethanol, propylene glycol, polyethylene glycol and glycerine, to aqueous systems is routinely used as an

aid to the solubilization of drugs in aqueous vehicles. In some other cases, the use of appropriate co-solvents can increase the aqueous solubility of a drug considerably. In other cases, the solubilizing effect is much less significant and may even lead to drug precipitation (Yalkowsky *et al*, 1981). Despite their popularity and utility in pharmacy, there are few systematic approaches for selecting co-solvents and predicting their solubilization effects on drugs. The selection of the appropriate co-solvent system can ensure the solubility of all formulation components and minimize the potential for precipitation, which may result from cooling or from dilution with body fluids. A straightforward and reliable approach that requires little or no experimental and thus minimal time and drug, is the log-linear model proposed by Yalkowsky and co-workers (1972, 1976, 1981). The log-linear model describes an exponential increase in nonpolar drug solubility with a linear increase in co-solvent concentration, and can be used to estimate drug solubility at high co-solvent concentrations. This relationship is described by:

$$\mathbf{Log\ S_{tot} = log\ S_w + \sigma * f_c} \qquad \text{eq 3.1}$$

Where S_{tot} is the total solute solubility in the co-solvent-water mixture, S_w is its water solubility, σ is the co-solvent solubilization power for the particular co-solvent-solute system, and f_c is the volume fraction of the co-solvent in the aqueous mixture. The σ term can be obtained from the slope of the log (S_{tot}/ S_w) versus co-solvent volume fraction (f_c) profile of selected drug and co-solvent.

These co-solvents have hydrogen bond donor and/or acceptor groups as well as small hydrocarbon regions. Their hydrogen bonding groups ensure water miscibility while their nonpolar hydrocarbon regions interfere with water's hydrogen bonding network. By disrupting self-association, they reduce water's ability to "squeeze out" nonpolar compounds and thus increase solubility.

Co-solvents can interact with water *via* two mechanisms. The organic nature of water-miscible co-solvents would lead to one type of interaction with water, which has been described, by the terms "iceberg" formation or "hydrophobic hydration" (Franks *et al*, 1966). Since this phenomenon depends on the ability of water to form cohesive bonds with other water molecules, it would be expected to be prevalent at relatively low concentrations of co-solvent, where an excess of water molecules exist. This phenomena has been reported in a number of investigators in aprotic co-solvents: water mixtures and in short-chain alcohol: water mixtures (Kimura *et al*, 1975). In addition to hydrophobic effects, the polar groups of co-solvent molecules lead to interactions with water of the hydrogen-bonding type. It has been suggested that low concentrations of relatively polar co-solvents, such as short-chain alcohols, may enhance water structuring through hydrogen bonding interactions in addition to the hydrophobic effect. Interactions between water and co-solvent which result in an increase in solvent structuring have been shown to reduce solvent-solute interactions. When the fraction of co-solvent is high relative to water, hydrogen bonding between water and co-solvent should also occur, but the significant structuring of water would be lost (Rubino *et al*, 1991).

Micellization and solubilization

Surfactant molecules consist of two moieties, one with affinity to water and the other to oil. When added to a mixture of oil and water, they self-assemble at the oil-water interface, so that the hydrophilic block stays in water and the hydrophobic one remains in oil. As the surfactant concentration increases, the monolayer at the oil-water interface becomes more densely populated and the interfacial tension decreases. At a certain concentration of surfactant, molecules start to self-associate in the bulk of one of the phases, forming supramolecular aggregates, (*i.e.* often spherical micelle in water) after reaching the critical micellar concentration (CMC). Above the CMC the concentration of free surfactant is not high regardless of the amount added because the surfactant molecules are aggregated into micelles (Kabalnov *et al*, 1996).

Nonionic surfactants can be prepared for example by reacting ethylene oxide with long chain hydrocarbons with terminal polar groups *e.g.* -OH, -COOH. This procedure introduces ethoxy groups, which are polar in nature and form hydrogen bonds with water. It increases the solubility of nonionic surfactants in water. The resulting molecules still have amphipathtic character and micelle formation takes place. In the micelle, the hydrophilic groups are orientated outwards and the lipophilic groups are inwards in aqueous solution. The CMC for non-ionic surfactants is, in general, low because of the absence of the electrical repulsive forces which are expected to oppose the micellization of ionic surfactants. Therefore, most of the surfactant is in the micellar state even in dilute aqueous solution. Surfactants in solution tend to form micelles, aggregates of colloidal dimensions existing in equilibrium with molecules or ions from which they are formed. One of the most important properties of micellar systems is their ability to solubilize hydrophobic drugs. For aqueous micelles, solubilization is closely related to the hydrophobic and amphithatic properties of solubilizate. One of the important roles for micelles is their use as reservoirs of monomers (Mittal, 1977a). Solubilization in micellar systems offers an attractive way of formulating poorly soluble drugs. For conventional polyoxyethylenated non-ionic surfactants maximal solubilization generally occurs at a hydrocarbon chain length of C_{16} atoms. An increase in chain length and decrease in the number of polyoxyethylene units indicate no increase in solubility, because when the alkyl chain length exceeds C_{16}, the chain is no longer liquid at 298 K and some of the polyoxyethylene glycol in order to depress its melting point and maintain a liquid micellar core (Lawrence, 1996).

Solubilization in the presence of surfactant (micellization) was defined by McBain *et al,* (1955) 'as spontaneous passage of insoluble molecules in water into an aqueous solution of a surface active agents in which thermodynamically stable solution is formed into fine particle' (reviewed by Elworthy, Florence and Macfarlane, 1975). Organic solutes can be solubilized by incorporation into surfactant micelles. The more non-polar the solute, the more likely it is to be incorporated near the core of the

micelle. The relationship between drug solubility in a micellar solution and surfactant concentration is described by the following equation:

$$S_{tot}^{mic} = S_w + k(C_{surf} - CMC) \qquad \text{eq. 3.2}$$

Where C_{surf} is the concentration of micellar surfactant (*i.e.* the total concentration minus the critical micellar concentration), S_w is the concentration of solute in pure aqueous solution, CMC is the critical micellar concentration of surfactant, and k is the molar solubilization capacity, the number of moles of solute that can be solubilized by 1 mole of micellar surfactant (Yalkowsky, 1999).

The exact location in the micelle at which solubilization occurs (the centre of solubilization) varies with the nature of the drug solubilized which reflects the interaction between the drug and the surfactant. Large polar drugs are believed to be solubilized in the aqueous medium between the individual molecules of surfactant in the palisade layer with the polar groups of the solubilizate oriented toward the polar groups of the surfactants and the nonpolar portions oriented toward the interior of the micelle. Interaction is predominantly by H-bonding or dipole-dipole attraction between the polar groups of solubilizate and surfactant. The extent of penetration in the palisade layer depends on the polar to nonpolar structures in the solubilizate molecules; longer-chain and less polar compounds penetrating more deeply than shorter-chain and more polar materials.

Several parameters could affect micelle formation and consequently solubilization, *e.g.* addition of salts, co-surfactant and changing pH. The addition of salts, in general, increased the solubilization but decrease the CMC, depending on the amount of the salt added. Addition of salt decreased the concentration of monomers present, thereby increasing the concentration of surfactant present as micelles, with a concomitant increase in the solubilizing power (McBain *et al*, 1941). The change in the CMC of non-ionic surfactants on the addition of electrolyte has been attributed

mainly to "salting out" or "salting in" of the hydrophobic groups in the aqueous solvent by the electrolyte, rather than to the effect of the latter on the hydrophilic groups of the surfactant (Elworthy, Florence and Macfarlane, 1975). When the monomeric form of a surfactant is salted out by the presence of an electrolyte, micellization is favoured and the CMC of the surfactant is decreased; when the monomeric is form is salted in, the CMC is increased. The hydrophobic group in the monomer phase is most likely affected by the addition of the electrolyte to the aqueous phase since the hydrophilic groups of the surfactant are in contact with the aqueous phase in both monomeric and micellar forms of the surfactant, while the hydrophobic groups are in contact with the aqueous phase only in the monomeric form. Therefore, the effect of the electrolyte on the hydrophilic groups in the monomeric and in the micellar forms may cancel each other (Elworthy, Florence and Macfarlane, 1975). Florence (1981) stated that the addition of sodium chloride to a mixture of surfactant and water alters the boundaries of the phases, possibly by altering the degree of hydration of the polyoxyethylene head groups of the nonionic surfactant. Interaction of solubilizate with the micelle may lead to changes in the packing of the monomer. There would be a shift in the phase boundaries not only of the isotropic micellar solution but also of the boundaries of mesomorphic phases, some of which disappear when disrupted by added solute because of the more delicate balance of forces in these systems.

Adding co-surfactant may alter the size and shape of micelles, and may increase the amount of solubilized oil in the system. Addition of a surfactant with low HLB limits the total surfactant solubility and thus the usefulness of the system, but small amounts of a hydrophobic detergent will sufficiently alter the micellar properties to result in changes in solubilization (Florence, 1981).

The solubilities of hydrophobic drugs in aqueous dispersions of SEDDS formulations are poorly understood and documented. Hydrophobic drugs are absorbed by transmembrane diffusion from the aqueous lumen of the gut. Most of the

hydrophobic drugs will have low solubility in the lumen, so the aim in practice should be to provide a reservoir of drug in a readily available form. Consequently, hydrophobic drugs may benefit from reformulation in lipids, which can provide a reservoir of drug dissolved in either lipid or micellar solution. Type I or type II lipid formulations are good candidates to avoid precipitation of hydrophobic drugs. However, these systems have limited solvent capacity for drugs such as cyclosporin A, which has encouraged the use of type III systems (Pouton, 1999). Type III systems upon dilution will form supersaturated solutions, but this need not necessarily lead to a rapid precipitation. The key issue is that if the drug precipitates on dilution of the formulation in the gut, then any advantage is likely to be lost.

It will be important for future formulations to establish methods for assessment of precipitation and predictive methods to prevent problems associated with solubility experiments. It is appropriate to consider the likely fate of each type of lipid formulation at this stage. Self-emulsifying systems are expected to disperse rapidly within the contents of the stomach, which indicates that the formulation will empty into the intestine in a manner similar to the emptying of aqueous solutions. Consequently, the rate of absorption from a type II and a type III system is likely to be rapid, particularly when administered to a fasted stomach. Hypnotics and analgesics are ideal to be formulated in these formulations because they require rapid onset of action. On the contrary, these formulations may be disadvantageous for a drug with low therapeutic index. Type I formulations are likely to be dependent on digestion by lipolysis, is a process which takes place in the small intestine. The drug will be solubilized in mixed micelles of bile salts as long as the drug does not precipitate during digestion. Therefore, the drug will not have an unusually rapid onset of action. The most important factor is the digestibility of the formulation. If the formulation is non-digestible then a colloidal state must be provided by self-emulsification, whereas if digestible oils are used, the colloidal state can be obtained by natural digestion. The overall bioavailability is equivalent to that achieved by type II or type III.

In this work, the factors which affect the precipitation of drugs on dissolution of lipid formulations, are investigated. At one extreme, when a hydrophobic drug is formulated in an aqueous co-solvent (such as propylene glycol or PEG 400), there is usually a drastic loss of solvent capacity and consequent precipitation of drug. At the other (lipophilic) extreme, if a drug is formulated in a triglyceride (TG) oil there will be no dissolution of TG, and no precipitation of drug. It can be anticipated that self-emulsifying formulations may also lead to a loss of solvent capacity, depending on the extent to which water-soluble components are used. Thus, type II SEDDS and S-F formulation should be less susceptible to precipitation than type III SEDDS.

The inclusion of hydrophilic surfactants and co-solvents could lead to precipitation depending on the proportion of water–soluble components used. If the formulation contains a high proportion (Type III B) then precipitation is to be expected. Whether this becomes a problem will depend on the rate of crystallization in the gut. This aspect of lipid formulation has not been studied thus far. In this chapter the approach that has been taken is to determine the equilibrium solubility of model drugs in dispersed systems. This is intended to help predict the likelihood of precipitation after administration of lipid systems. Therefore, steroids were chosen to give a range of log *P* because we expected that some would precipitate but others would stay in solution on dilution.

3.2 Materials and methods

3.2.1 Materials

In addition to the materials mentioned in chapter 2, the following materials used:

Trizma®-maleate (tris-maleate)	$C_4H_{11}NO_3C_4H_4O_4$	50 mM	Sigma
Sodium chloride	NaCL	150 m	Sigma
Calcium chloride dihydrate	$CaC_{12}.H_2O$	5 mM	Sigma
Sodium hydroxide	NaOH	qs	Fisons

3.2.2 Methods

3.2.2.1 Solubility Measurements

An excess amount of steroid powder was added to 20 ml glass vials containing various percentages (0.1 % w/v-4.0 % w/v) of hydrophilic co-solvents, lipophilic surfactants and dispersed SEDDS formulation (Ch.2, [Table 2.4]). Triplicate sample vials prepared for each lipid excipients and/or SEDDS formulation in water or tris-maleate buffer (pH 6.5) and were placed on an end-over-end shaker water bath at room temperature for 48 hour. Samples with drug were considered to reached equilibrium after 2 days and removed from the shaker. The samples were then centrifuged using Jouan 3.11 centrifuge (Decon laboratories, Ltd, model # FS200B, UK) at 3230 g for 20 min. Supernatant was diluted using methanol 96%v/v before measured the absorbance using the UV Spectrophotometer. The concentration of the drug was calculated as mentioned in (Ch.2, [2.2.2.3 UV assay].

Table 3.1 summarizes the lipid excipients and SEDDS formulation used in this investigation

	Hyd.		H.A		Tes.		T.A		Prog.	
	H_2O	tris-Ma.	H_2O	tris-Ma.	H_2O	tris-Ma.	H_2O	tris-Ma.	H_2O	tris-Ma.
PEG400	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
PG	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
CRH 40®	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
Tween 80®	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
Miglyol 812®	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
S-F (M812®+I988®+ PG) (30%+50%+20%)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
Type II (M812®+TTO®) (40%+60%)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
Type IIIA (M812®+I988®+CRH 40®) (30%+30%+40%)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
Type IIIB (M812®+I988®+CRH40®+PG) (10%+10%+40%+40%)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔

Table 3.1 Lipid excipients and SEDDS formulation used to assess the fate of steroids in water and/or tris-maleate buffer (pH 6.5) at 25°C. The dilution was used 1g excipients in 25 ml (4 %w/v) of water and/or tris- maleate buffer (pH 6.5) (✔ indicates that the experiment were done).

3.3 Results and discussion

The solubility values of steroid derivatives (g%w/v) in diluted hydrophilic co-solvent, lipophilic surfactant and SEDDS formulations (% w/w) are presented in appendices 2, Tables 2.1-2.9. Experiments were performed in triplicate (SD <2% of mean).

3.3.1 Cosolvency

Yalkowsky *et al*, (1981) has shown that the solubilization capacity σ is dependent upon the molecular hydrophobic surface area of the solute and on the interfacial tension of the pure co-solvent. The solubilizing power (σ) for the drug is dependent upon the drug polarity, measured by log *P* Co/W, and can be calculated from equation 3.1 (by the linear regression of the log solubility (% w/v) plot of the data. The dependence of σ on log Pc on the solubilization for steroid derivatives is shown in Table 3.2. The more hydrophobic the drug (log *P*) the greater the solubilization capacity. Although some exceptions were observed in case of testosterone and its acetate ester. The solubilizing capacity increased from 0.284 for hydrocortisone (log *P* 1.61) to 0.4864 for testosterone acetate (log *P* 4.7) in aqueous solution of 4%w/v PG. The same trend was observed for aqueous solution of 4%w/v PEG 400. The solubilizing capacity increased from 0.247 for hydrocortisone to 0.264 for progesterone. Testosterone and its acetate ester produced a lower solubilizing capacity than expected in the aqueous solution of PEG 400 0.112 and 0.1306 respectively due to an experimental error.

The solubilizing capacity of steroids in tris-maleate buffer (pH 6.5) of 4 %w/v PEG 400 showed an increase from 0.260 for hydrocortisone to 0.466 for progesterone. The same trend was observed for tris-maleate buffer (pH 6.5) of 4 % w/v PEG 400 for hydrocortisone and its acetate ester 0.381 and 0.155 respectively. In case of progesterone, testosterone and its acetate ester indicated a decrease in solution of PG 0.4579, 0.1453 and 0.235 respectively. These unexpected measurements as a result

of the solubility of the drug in dispersed co-solvent did not reach saturation in some cases. Furthermore, there may have been problems associated with obtaining homogenous samples of the dispersion upon centrifugation.

Usually, co-solvents form a homogenous solution with water. These solutions act as modified solvents having a polarity between that of water and the pure co-solvent (Yalkowsky, 1999). In any co-solvent mixture, the concentration of the unionised species and the ionized species in equilibrium. The unionised species, in general, is often assumed to be primarily responsible for improvement in total drug solubility. Figures 3.1- 3.5 show the solubility measurements obtained for steroids in propylene glycol and/or polyethylene glycol 400 dispersed into water and/or tris-maleate buffer pH 6.5. These data indicate a general increase in solubility as the weight fraction of co-solvent in the mixture is increased. The solubility increases are greater for more hydrophobic drugs. Although the data indicated an increase in solubility when the weight fraction increased, it is apparent that plots are not linear. At low weight fraction of co-solvent, the solubility of steroids was low.

A possible explanation for the low solubility can be obtained from the work of Kimura *et al.* who observed similar patterns of deviations in heat of solutions data of lipophilic solutes in alkanol: water mixtures (Kimura *et al*, 1975). At low concentrations of co-solvent water retains its ability to form highly ordered structures. Co-solvents possess both polar and nonpolar groups and it is expected that hydrophobic hydration occurs around nonpolar portions of the molecule, while hydrogen bonds are formed between polar groups and water. Such interactions between water and co-solvent reduce the interaction between water and the solute molecules, resulting in a solubility that is lower than is expected (Rubino *et al*, 1991). The interaction between water and a more lipophilic drug would be more highly dependent on the availability of water molecules in order to maintain the solute in solution due to the lower density of hydrogen bonding groups on the solute as seen in case of hydrocortisone and its salt. Thus, the more lipophilic species of the drug

series might more sensitive to the co-solvent–water interactions and this could explain the apparent correlation between observed solubility and steroids series. In addition, Arnett and McKelvey (1965) reported that endothermic shifts due to hydrophobic hydration are dependent on the size of the solute. In other calorimetric studies systems, in which a single solute is examined in binary mixtures of various short chain alkanols and water, the magnitude of the co-solvent is similarly related to the alkyl chain length of alkanol. At high concentrations of co-solvent, the three–dimensional structure of water is lost and water molecules would be more available to interact with solute molecules; this leads to greater solubilites.

Log solubilization curves of a series of steroids in PG and PEG 400 in H_2O and/or tris-maleate buffer pH 6.5 at 25 °C are shown in Figures 3.1-3.5. The solubilization curves show similar pattern for all steroids but a different patterns in the quantity dissolved.

The intrinsic solubility (S_i) of testosterone and its acetate ester in pure PG was 0.828g % w/v and 0.876 g % w/v, respectively then the maximum dose, which can be administered in solution, is approximately 8.28 mg / ml and 8.76 mg/ ml, respectively *i.e.* 8.28mg in 1 ml capsule and 8.76 mg in 1 ml capsule, respectively. This means that a hydrophilic co-solvent of 1 g diluted in 100ml must be able to dissolve the drug at 8.28 mg / 100ml (0.0828 g % w/v). The solubility of testosterone and its acetate ester at 1 g % w/w aqueous solution of PG was 0.0113 g % w/v and 0.109 g % w/v, respectively. This indicates that there would be loss of the original quantity <40% of testosterone leading to precipitation but still the co-solvent can keep some of the drug in solution. Progesterone has similar trend, the intrinsic solubility is 1.071g%w/v, so the maximum dose is approximately 10.071mg/ml, this mean a 1 g of hydrophilic co-solvent diluted to 100 ml must be able to dissolve the drug at 10.071 mg/100 ml *i.e.* 0.01 g%w/v and at 1%w/v aqueous solution of PG was 0.0063g% w/v. However, hydrocortisone acetate exhibited a significant loss in aqueous solution of PG 0.0068 g%w/v given the intrinsic solubility is 0.337g%w/v *i.e* the maximum dose is

approximately is 3.37mg/ml, this means 1g of hydrophilic co-solvent diluted to 100 ml must be able to produce the drug at 3.37mg/100 ml (0.0337g%w/v). The intrinsic solubility of hydrocortisone is 1%w/v *i.e.* the maximum dose which can be administered in solution, is approximately 10 mg/ml, this means that 1g of hydrophilic co-solvent diluted to 100 ml must be able to dissolve the drug at 10 mg/100 ml *i.e.* 0.01%w/v, the results shows that the aqueous solubility of 1g of PG diluted in 100 ml water was able to dissolve 0.0113%w/v *i.e.* 11.4 mg/100 ml, this results indicated that co-solvent can keep hydrocortisone into solution higher than expected.

Based on the data shown, PEG 400 indicated better ability to keep the drug in solution than PG. Figures 3.1 and 3.2 showed that testosterone and its acetate ester exhibited better solubility at 1 %w/v of aqueous solution of PEG 400 or in tris-maleate buffer pH 6.5 0.0328 g%w/v, 0.054 g%w/v; 0.1075 g %w/v and 0.1280g% w/v respectively, than at 1% w/v aqueous solution of PG or in tris-maleate buffer (pH 6.5) 0.0113 g%w/v, 0.0715g% w/v; 0.109 g%w/v, and 0.095g% w/v respectively. Figure 3.3 showed that progesterone followed the same trend; in the presence of 1%w/v of aqueous solution and/or of PEG 400 or tris-maleate was 0.0141 g % w/v and 0.0940 g % w/v, respectively, while in the presence of PG the solubility was 0.0063 g % w/v and 0.0225 g % w/v respectively. Figure 3.4 showed that hydrocortisone acetate follow similar pattern to the other steroids. However, in Figure 3.5 showed that hydrocortisone exhibited higher solubility in the presence of PG in water and/or tris-maleate buffer 0.0103 g%w/v and0.0153 g%w/v respectively than in the presence of PEG 400 either in water or in tris-maleate buffer (pH 6.5) 0.0113g%w/v and 0.0109g%w/v respectively.

Solubilization power σ				
	PG	PG	PEG 400	PEG 400
	H2O	Tris-maleate	H2O	Tris-maleate
Hydrocortisone	0.284	0.381	0.247	0.260
Hydrocortisone acetate	0.127	0.155	0.288	0.298
Testosterone	0.394	0.145	0.112	0.262
Testosterone acetate	0.486	0.235	0.131	0.193
Progesterone	0.465	0.458	0.264	0.466

Table 3.2 The solubilization power of steroids either in water or in tris-maleate buffer (pH 6.5) at 25 °C of 4 % w/v PG and/or PEG 400 solution (σ is the linear regression of the log plot of the data).

3.3.2 Micellization and solubilization

Solubilization by surfactant is generally believed to be *via* uptake of solute molecules into isotropic solutions of surfactants (Florence, 1986). Two surfactants, Cremophor RH 40® (CRH 40®) and Tween 80® (T 80®), were used as representative non-ionic surfactants. They were chosen on the basis of their polyoxyethylene chain length. Tween 80 (polyoxyethylene-20-sorbitan monooleate) is a clear, yellow liquid at room temperature with HLB = 15. Cremophor RH 40® (polyoxyl-40-hydrogenated castor oil) is white solid material at room temperature with HLB = 14-16.

Surfactant	CMC
Cremophor RH 40	0.0039
Tween 80®	0.000021

Table 3.3 The critical micellar concentration of non-ionic surfactant used in the research (Samah *et al*, 1989).

In general, both CRH 40® and T 80® produce similar effect on steroids solubility. These effects for all surfactant-steroid systems are given in Figures 3.6-3.11. The amount of drug solubilized (g/100ml) was linearly related to surfactant concentration (% w/v) ($r^2 = 0.98 \pm 0.5$), either in water or in tris-maleate buffer pH 6.5 at 25°C. The

y-x intercept equated to steroid's aqueous solubility indicating that its solubility at the CMC was very close to that in water. This is expected since the CMCs of surfactant were very low when compared with the surfactant concentration used (Barry *et al*, 1976). In Figure 3.9, the aqueous solubility of hydrocortisone at 0.1 % w/w CRH 40® in water is 0.2 g /ml and this is very close to its solubility in water (0.25 g/ml). When surfactant concentrations were compared on a weight basis, the solubilizing efficiency of the surfactants, decreased with increasing polyoxyethylene chain length. The aqueous solubility of steroids increased in water in the presence of CRH 40® or T 80® as the polarity of the steroids increased *i.e.* increased log *P*.

In the presence of tris-maleate buffer pH 6.5, the aqueous solubility of steroids increased more in the presence of T80® than in the presence of CRH 40®. Ethoxylated surfactant owing to the absence of electric charges, are insensitive to pH and practically unaffected by salt concentration (Becher, 1983a). Nevertheless, the addition of some electrolytes will change the orientation of the water molecules on the surface giving a positive surface potential. With increasing salts concentration, non-ionic surfactant lose part of the water of hydration. This is a salting out effect and will lead to a decrease in the CMC of the surfactant. The salting out of neutral molecules depends on the concentration and the ionic radius of the added electrolytes. The smaller the hydrated ions the bigger the salting out effect because of the stronger polarizing effect. Addition of electrolytes decreases hydration of the ether linka 96 non-ionic surfactant and shifts the hydrophilic-lipophilic balance to the lipophilic site (Lissant, 1974).

Steroids are non-electrolytes at neutral pH (6.5); the drug is primarily uncharged and the solubilization by both surfactants is approximately similar. Tween 80® solutions exhibited higher solubility in tris-maleate buffer pH (6.5) than in the presence CRH 40®. Figures 3.6 and 3.7 showed that testosterone and its acetate ester exhibit better solubility in the presence of 1%w/v T80® in tris-maleate buffer 0.3781 %w/v and 0.3375 %w/v) than in the presence of CRH 40® 0.3425 %w/v and 0.3075%w/v

respectively. Figure 3.8 showed that hydrocortisone acetate followed the same order, better solubility in the presence of 1%w/v T 80® in tris-maleate buffer pH (6.5) 0.211 %w/v than in the presence of CRH 40® 0.200%w/v respectively. On the contrary, Figures 3.9 and 3.10 showed that hydrocortisone and progesterone had better solubility in the presence of 1 % w/w CRH 40® of tris-maleate buffer pH (6.5) 0.1695 % w/v and 0.2085 % w/v, respectively than in the presence of T 80® 0.1400 %w/v and 0.1778 % w/v respectively. Results revealed that the micellar sizes of surfactants decrease while hydration increases as polyoxyethylene chain increases and so, inclusion of non-polar, non-electrolyte steroids into the increasingly polar micellar environment decreased (Barry *et al*, 1976).

The intrinsic solubility of hydrocortisone in CRH 40® was 0.73 %w/v, then the maximum dose which can be administered in solution is approximately 7.3 mg/ml; *i.e.* 7.3 mg in 1 ml capsule. This means that a 1g of CRH 40® diluted in 100 ml water must be able to dissolve that hydrocortisone at 7.3 mg/100 ml (0.073 %w/v). The aqueous experiments revealed that both surfactants could keep drug into solution higher than expected. The same trend happened with testosterone and its acetate ester, the aqueous solubility was much higher than expected. The intrinsic solubility in CRH 40® was 0.97 %w/v and 1.06 %w/v, respectively. The maximum dose which can be administered in solution is approximately 9.7mg/ml, 10.6mg/ml, respectively. This means that a 1 g of CRH 40® diluted in 100 ml water must be able to dissolve that testosterone and its acetate ester at 9.7mg/100ml (0.097%w/v), 10.6 mg/100ml (0.106%w/v), respectively. Progesterone has similar behaviour; the intrinsic solubility in CRH 40® was 1.21%w/v. This means that the maximum which can be administered in solution is approximately 12.1mg/ml (12.1mg in 1 ml capsule) *i.e.* 1 g of CRH 40® diluted in 100 ml water must be able to dissolve that progesterone at 12.1mg/100 ml (0.121%w/v). The data indicated that a saturated solution in pure T80® or CRH 40® would stay in solution on dilution.

Different opinions were describing the hydrated polyoxyethylene chain. Schick (1963) has been proposed that polyoxyethylene chain is an expanding spiral *i.e.* a cone shape, with the narrower end at the surface of hydrocarbon core. Later, other researchers mentioned that the micellar structures of these surfactants were nearly spherical and extensively hydrated (reviewed by Elworthy *et al*, 1975). Although there is space for hydrating water in the outer parts of the micelles, there is virtually none close to the hydrocarbon core due to crowding of the polyoxyethylene chains (Elworthy *et al*, 1975). This produces a region which is largely purely polyoxyethylene, rather than polyoxyethylene-water, which may act as a site of solubilization of semi-polar drugs. A cross section of a polyoxyethylene surfactant micelle therefore offers a complete range of polarity. The non-polar hydrocarbon core will be, *via* the semi-polar polyoxyethylene portion, to pure water on the micellar surface. For semi-polar steroid drugs, it would be likely that the unhydrated polyoxyethylene portion close to the hydrocarbon core, so that the least polar steroids, testosterone acetate>progesterone> testosterone, would be closest to the core and the polar ones, hydrocortisone and hydrocortisone acetate, furthest from the core and closer to the hydrated polyoxyethylene portion (Barry *et al*, 1976).

3.3.3 The fate of SEDDS formulation upon dilution

Lipid formulations are considered when the poor aqueous solubility of a drug candidate is attributed to its hydrophobicity (Anderson *et al*, 1999). For lipid formulations it is necessary to understand how solubility is affected by mixing different components, such as medium chain oils, hydrophilic co-solvents and non-ionic surfactants and how solubility is related to the molecular weight, melting point and log *P* of the drug. It is useful to be able to anticipate whether precipitation of the drug is likely to occur after dispersion of the formulation in gut. The solubility of each steroid was determined in aqueous dispersions of SEDDS formulations either in water or in tris-maleate buffer (S) pH 6.5. This data was used to predict whether a saturated solution of drug in the undiluted formulation would remain in solution (at

equilibrium) in dilutions of 1 in 25 ml. The mass (mg) of each steroid dissolved in the aqueous dispersion (4 % w/v) was estimated from experimental data and tabulated in Tables 3.4 and 3.5.

The mass of steroid in 1 g pure formulation was calculated from the data described previously on intrinsic solubility (S_i), as shown in Table 3.4.

Type I formulations show, in general, the extent of solubility of aqueous dispersions of the SEDDS formulations increased with the hydrophobic character of steroids. Type I systems are represented by using Miglyol 812® as medium chain fatty acid oil. The date in Table 3.5 showed that the intrinsic solubility of steroids in Miglyol 812® is limited. The intrinsic solubility of progesterone, testosterone, and its acetate ester are 1.147 % w/v, 0.847 % w/v and 1.035 % w/v respectively. The data in Table 3.4 showed that 1 g of pure M 812® diluted to 100 ml water and/or tris-maleate buffer (pH 6.5) must be able to dissolve 11.47 mg, 8.470 mg, and 10.35 mg per 100 ml, respectively. However, in fact the data showed that the mass recovered was much less than expected 6.91 mg, 3.49 mg, and 4.67 mg respectively when diluted into water and 3.99 mg, 2.86 mg and 3.81 mg respectively when diluted into tris-maleate buffer. The same trend was observed for hydrocortisone and its acetate ester form. The hydrocortisone formulation was soluble at 0.37 mg and 0.271 mg respectively after dilution in either water or tris-maleate buffer respectively. The corresponding figures for hydrocortisone acetate were 2.2 mg and 1.41 mg respectively. The experimental data indicated an unexpected result that precipitation of the drugs may occur after dilution of type I formulations in water or tris-maleate buffer. Normally, type I (MCT) would be expected to keep the drug in solution upon digestion, since medium chain glycerides will undergo lipolysis, which facilitates dispersion of the drug into colloidal solution. However, if the dose of the drug is low, as in steroids, the formulator may be uncertain of the fate of the drug.

	mass(mg) in 1 g pure form.	mass(mg) Type I H_2O	mass(mg) Type I tris-m.	mass(mg) in 1 g pure form.	mass(mg) Type II H_2O	mass(mg) Type II tris-m.	mass(mg) in 1 g pure form.	mass(mg) Type III A H_2O	mass(mg) Type III A tris-m.	mass(mg) in 1 g pure form.	mass(mg) Type III B H_2O	mass(mg) Type III B tris-m.	mass(mg) in 1 g pure form.	mass(mg) S-F H_2O	mass(mg) S-F tris-m.
H	1.000	0.370	0.271	1.760	0.290	0.310	7.770	2.680	2.590	10.890	2.100	2.310	12.030	4.900	4.560
H.A	3.860	2.211	1.020	0.480	0.141	0.123	2.060	0.520	0.630	3.000	0.430	0.449	1.632	0.651	0.563
T	8.470	3.490	2.860	7.600	3.010	3.750	10.340	3.600	2.790	11.300	2.000	2.270	25.660	12.300	9.190
T.A	10.350	4.760	3.810	6.690	3.300	3.560	11.040	3.560	2.990	8.400	1.990	1.870	20.600	8.630	9.210
P	11.470	6.910	3.990	6.100	2.420	2.420	13.640	4.250	4.310	14.890	2.890	2.789	22.150	8.470	9.170

Table 3.4 The mass (mg) of steroids in 4 g formulation either diluted in 100 ml of water or in tris-maleate buffer (pH 6.5) and the mass of steroids in pure formulations (1 g).

	Log P	Type I S_i	S %w/v H_2O	S %w/v tris-m	Type II S_i	S %w/v H_2O	S %w/v tris-m	Type IIIA S_i	S %w/v H_2O	S %w/v tris-m	Type IIIB S_i	S %w/v H_2O	S %w/v tris-m	S-F S_i	S %w/v H_2O	S %w/v tris-m
H.	1.53	0.100	0.037	0.027	0.176	0.029	0.031	0.777	0.268	0.259	1.089	0.210	0.231	1.203	0.490	0.456
H.A.	2.48	0.386	0.022	0.102	0.048	0.014	0.112	0.206	0.052	0.063	0.300	0.043	0.045	0.163	0.651	0.560
T.	3.30	0.847	0.349	0.286	0.760	0.301	0.283	1.034	0.375	0.279	1.130	0.020	0.227	2.566	1.230	0.919
T.A	4.70	1.035	0.476	0.381	0.669	0.330	0.301	1.104	0.356	0.299	0.840	0.199	0.187	2.060	0.863	0.921
P.	3.80	1.147	0.691	0.399	0.610	0.246	0.242	1.364	0.425	0.431	1.489	0.289	0.279	2.215	0.847	0.917

Table 3.5 The aqueous solubility of steroids at 4 % w/w SEDDS formulation in water and/or tris-maleate buffer pH 6.5 (S % w/v) and their intrinsic solubility (S_i g % w/v) at 25°C.

These unexpected measurements could be as a result of the solubility of drug in the dispersed formulations did not reach saturation in some cases. Furthermore, there may have been problems associated with obtaining homogenous samples of the dispersion upon centrifugation.

In type II system, the magnitude of the observed aqueous solubility of steroids in the dispersion of type II diluted either in water or in tris-maleate, can be ranked in the following order: testosterone acetate>testosterone > progesterone> hydrocortisone > hydrocortisone acetate. The expected mass of progesterone, testosterone, and testosterone acetate from dissolving 1 g of pure type II formulation to 100 ml was 6.10 mg, 7.6 mg and 6.69 mg respectively. The amount of mass recovered from progesterone, testosterone, and testosterone acetate was 2.46 mg, 3.01 mg, and 3.3 mg respectively when diluted into water. The amount of hydrocortisone and its acetate form mass recovered was 0.29 mg and 0.141 mg, respectively, which less than expected 1.76 mg and 0.48 mg respectively. The data indicated that at least 50% of the drug would be precipitated on dilution of a Type II system.

Type II systems are defined as self-emulsifying systems that are comprised of water-insoluble components. They are composed of non-ionic ester ethoxylated surfactant with an intermediate HLB 12 mixed with medium chain triglycerides. The type II mechanism could be described by the 'interfacial turbulence' mechanism. The process of emulsification is thought due to the occurrence of turbulence at the oil-water interface. The occurrence of turbulence is due to unequal adsorption and diffusion of surface-active materials at the interface. The unequal surfactant concentration leads to corresponding spreading of the interface, causing violent turbulence. This causes droplets of one phase to appear in the other along the oil-water interface. The surfactant forms a new oil-water interface around the droplets and emulsification occurs. They have the tendency to form lamellar liquid crystalline structures upon dilution, which facilitate penetration of water in formulation, resulting into interfacial disruption, which is strong enough to eject the emulsion

particles from the surface of the bulk oil-surfactant interface. From the data shown in Tables 3.4 and 3.5, the molecular weight of testosterone (Mwt=288.4) and its acetate ester (Mwt = 330.5) are appropriate in size to be dissolved within the interface, unlike hydrocortisone acetate (Mwt=404.3). The melting point may play a role in solubilization; testosterone and its acetate ester 152° and 140° respectively is lower than hydrocortisone and its acetate form 217°-220° and 220° respectively.

Table 3.5 showed the ability of hydrophobic drugs to stay in solution should improve upon moving from type II to type III systems. This is achieved by the inclusion of hydrophilic surfactants (such as Cremophor RH 40®). The intrinsic solubility of steroids in type III systems is better than in type II formulations. Therefore, the amount of mass expected to be dissolved in 1 g pure type III formulation diluted in 100 ml solution must be more than in type II and type I (Table 3.4). Upon dilution of Type II SEDDS in water, the hydrophilic co-solvent diffuses away from the oil into the aqueous phase, thus the mechanism is 'diffusion and stranding'. The hydrophilic surfactant may also transfer to the aqueous phase to form a micellar solution. The fate of hydrophobic drugs in type III systems, upon mixing with water could be solubilization in a swollen micellar solution, dispersion as a fine emulsion, or isolation and precipitation. For steroids, most of them had a precipitation problem.

Due to this confusion, type III system can be subdivided further into two types depending on the presence of surfactant. In type III B the hydrophilic content is higher than in type III A. Pouton (1999) mentioned that for drugs having an intermediate log *P*, the best formulation would be type III B, because the content of oil is less than 20 %. However, the results revealed that there was no specific relationship between aqueous solubility (4 % w/v) of the drug and its hydrophobic, molecular weight and melting point; rather it is drug dependent.

All steroids exhibit better solubility in dispersions of type III A than dispersions of type III B systems. The intrinsic solubility of testosterone and its acetate ester in type

III A was 1.034 %w/v and 1.1040 %w/v respectively and in type III B was 1.130%w/v and 0.84% w/v respectively. This means 1 g of type III A would dissolve 10.34 mg of testosterone and 11.04 mg for testosterone acetate. The mass recovered of testosterone either in aqueous dispersion of type IIIA or in tris-maleate buffer was 3.60 mg, and 2.79 mg respectively and 3.56 mg and 2.99 mg respectively for testosterone acetate. Either in the presence of type III B dispersed in aqueous solution or in tris-maleate buffer, the mass recovered was 2.00 mg and 2.27 mg, respectively for testosterone and 1.99 mg and 1.87 mg respectively for its acetate ester. This represents a loss of up to 80% of the drug, in spite of the presence of hydrophilic surfactant CRH 40®, <40 % which would be expected to solubilize the drug through the micellization. The presence of hydrophilic co-solvent <35 % would be expected to lead to precipitation of the drug, so the amount of mass recovered after dilution of type III B was less than in type III A.

The same basic observations applied to hydrocortisone and its acetate ester. The intrinsic solubility of hydrocortisone was 0.77 %w/v and 0.206 %w/v for hydrocortisone acetate. Therefore, 1 g of pure type IIIA should be able to dissolve 7.7 mg for hydrocortisone and 2.06 mg for the acetate ester. The mass recovered of hydrocortisone upon dilution type III A either in water or in tris-maleate buffer was 2.68 mg and 2.59 mg, respectively, and 0.52 mg and 0.63 mg respectively for the acetate ester. The amount of drug recovered in the presence of type IIIB was 2.10 mg, 2.31 mg, respectively for hydrocortisone and 0.43 mg; 0.44 mg respectively for the acetate ester.

Progesterone formulations behaved in a similar manner. The mass recovered in the presence of type III A upon dilution either in water or in tris-maleate buffer was more 4.25 mg and 4.310 mg respectively than in the presence of type III B 2.89 mg and 2.79 mg respectively.

The previous data was anticipated for the use of type III A and type III B formulations, however the mass recovered was much less than expected for the type I and type II formulations. This could have been due to experimental error, perhaps the time for equilibration was insufficient or the samples were not sufficiently homogeneous before and after centrifugation.

In S-F formulation, the presence of >20 % medium chain fatty acid oil and > 40 % of mono-, di-, and tri-glycerides may fulfil a valuable role in preventing precipitation of the drug on dilution in water. The presence of high oil content is usually desirable because it will be digested rapidly following its entry into the duodenum. In this way the increased concentration of drug in solution within mixed micelles enables rapid absorption in the upper small intestine (Hutchison *et al*, 1996). The data showed that in comparison with pure co-solvent formulations, S-F formulations helped to keep the drug in solution upon dilution either in water and/or in tris-maleate buffer. However, again, the amount of mass recovered was much less than expected due to the previous reasons mentioned. For example, the intrinsic solubility of hydrocortisone and its acetate ester in the formulation was 1.203 %w/v and 0.1632 %w/v respectively. Therefore, 1 g of pure S-F must be able to dissolve 12.03 mg in 100 ml for hydrocortisone and 1.632 mg in 100 ml for hydrocortisone acetate. The mass recovered for hydrocortisone upon dispersion of S-F either in water and/or tris-maleate was 4.90 mg and 4.56 mg respectively, and 0.651 mg and 0.563 mg respectively, for its acetate ester. The same followed for progesterone; the recovered mass was much less 8.47 mg in water and 9.17 mg in tris-maleate than the expected mass upon dilution S-F either in water and/or in tris-maleate buffer 22.15 mg. The expected mass of testosterone and its acetate ester upon dilution S-F was more 25.66 mg and 20.6 mg; respectively than the recovered upon dilution either in water and/or in tris-maleate buffer 12.3 mg and 9.21 mg respectively for testosterone 8.630 mg and 9.21 mg respectively for testosterone acetate.

3.4 Conclusion

Formulations selection is important in relation to the objective of keeping the drug in solution throughout its passage in the gut. The first estimation to make from the equilibrium solubilities is what happens in the expected worst case represented by formulation as a simple co-solvent solution. The average solubility of steroids in co-solvent is 1%w/v; therefore, the maximum dose that can be administered in solution is approximately 10 mg/ ml, *i.e.* 10mg in a 1ml capsule. This means that a formulation of 1g diluted to 100ml must be able to dissolve the drug at 10mg/100ml (0.01% w/v; 0.1mg/ml). The data indicated that at least 60% of the drug would be precipitated on dilution of a co-solvent.

Since the steroids are only soluble at approximately 1.1 % w/v in each formulation, then the maximum dose, which can be administered in solution, is approximately 11 mg/ ml, *i.e.* 11mg in a 1ml capsule. This means that a formulation of 1g diluted to 100ml must be able to dissolve the drug at 11mg/100ml (0.011% w/v; 0.11mg/ml). Type I formulation (Miglyol 812®) would be expected to keep the drug in solution upon dispersion and digestion better than type III A and III B. Surfactant-free formulations could be used to administer a higher dose of steroids since the steroids are soluble at approximately 2 % w/v. Then the maximum dose, which can be administered in solution, is approximately 20 mg/ml, *i.e.* 20mg in a 1ml capsule. This means that a formulation of 1g diluted to 100ml must be able to dissolve the drug at 20mg/100ml (0.02% w/w; 0.2mg/ml).

The aqueous solubility of drugs in dispersions of SEDDS formulations was not as high as expected but gave an idea of the minimum solubility of the drug after dispersion. Steroids were not very successful model drugs to examine the fate of drugs after dispersion, because of their low solubility in the formulations. Steroids are non-electrolytes that are poorly soluble in the lumen of the small intestine. Therefore, they may benefit from reformulation into lipid systems, which can give a

reservoir of drug dissolved in either lipid or micellar solution. The key issue is the hydrophilic content (surfactant and co-solvent) that is required to keep the drug in solution. In practice, the presence of co-solvent and surfactant can improve the solvent capacity of the formulation, but the instant they are diluted the fates of co-solvent systems will differ from surfactant systems. For co-solvent dispersions large amounts of the drug may precipitate whereas surfactants are capable of forming micellar solutions, which may be provide better solvents. The results presented here indicated that even in the presence of surfactant there is a possibility of loss of the drug from type III B formulations.

Figure 3.1 The fate of testosterone in water and/or tris-maleate buffer (pH 6.5) of hydrophilic co-solvent at 25°C represented by log solubility Vs hydrophilic co-solvent concentration (% w/v)

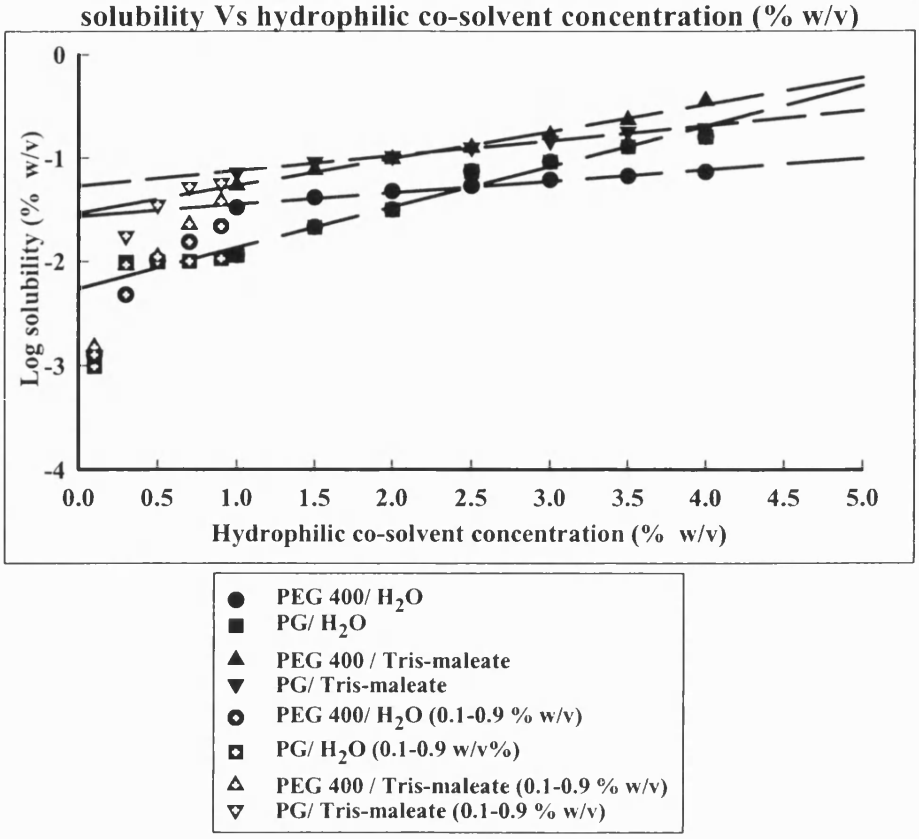


Figure 3.2 The fate of testosterone acetate in water and/or tris-maleate buffer (pH 6.5) of hydrophilic co-solvent at 25°C represented by log solubility Vs hydrophilic co-solvent concentration (% w/v)

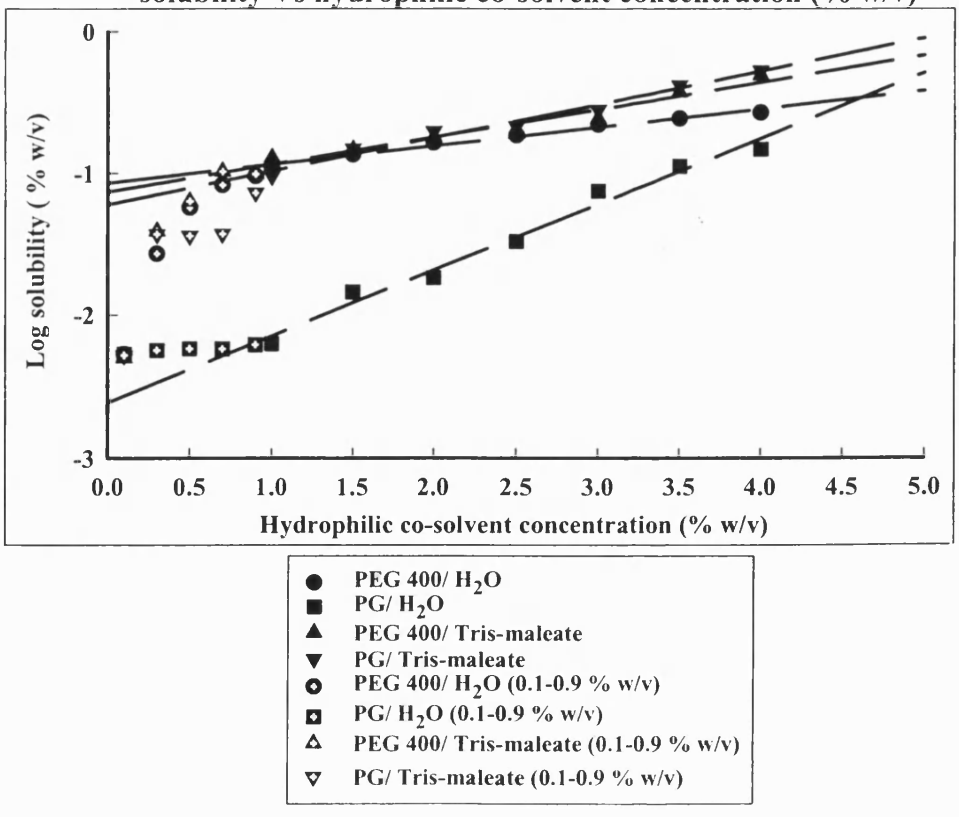


Figure 3.3 The fate of progesterone in water and/or tris-maleate buffer (pH 6.5) of hydrophilic co-solvent at 25°C represented by log solubility Vs hydrophilic co-solvent concentration (% w/v)

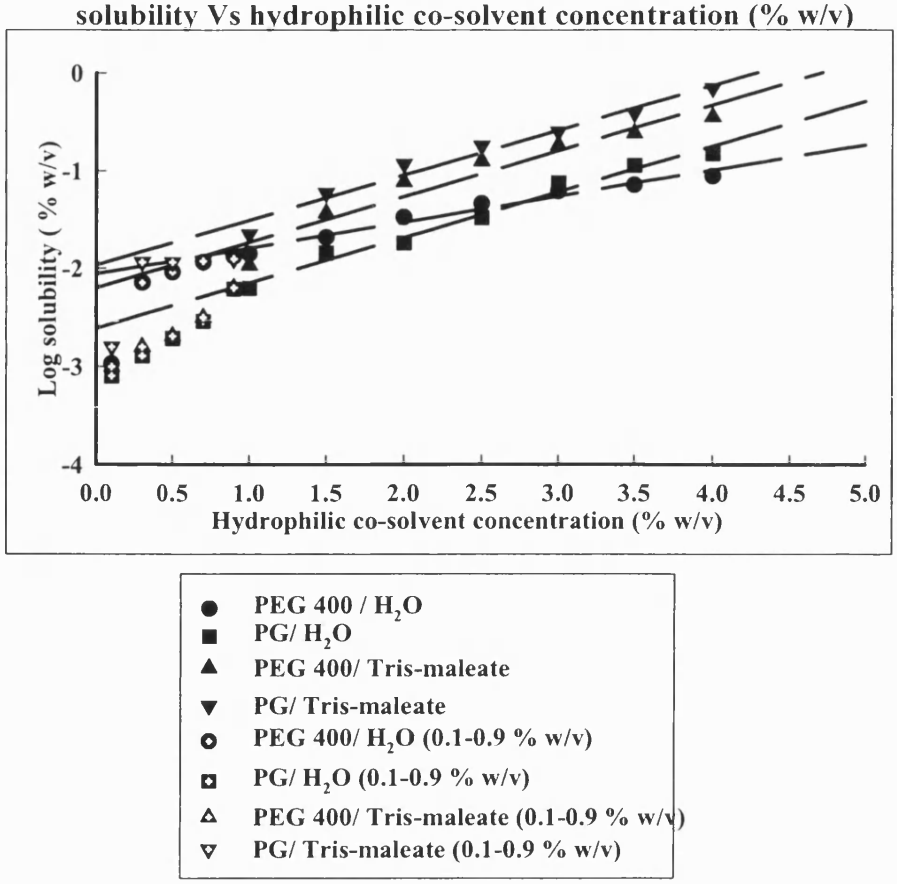


Figure 3.4 The fate of hydrocortisone acetate in water and/or tris-maleate buffer (pH 6.5) of hydrophilic co-solvent at 25°C represented by log solubility Vs hydrophilic co-solvent concentration (% w/v)

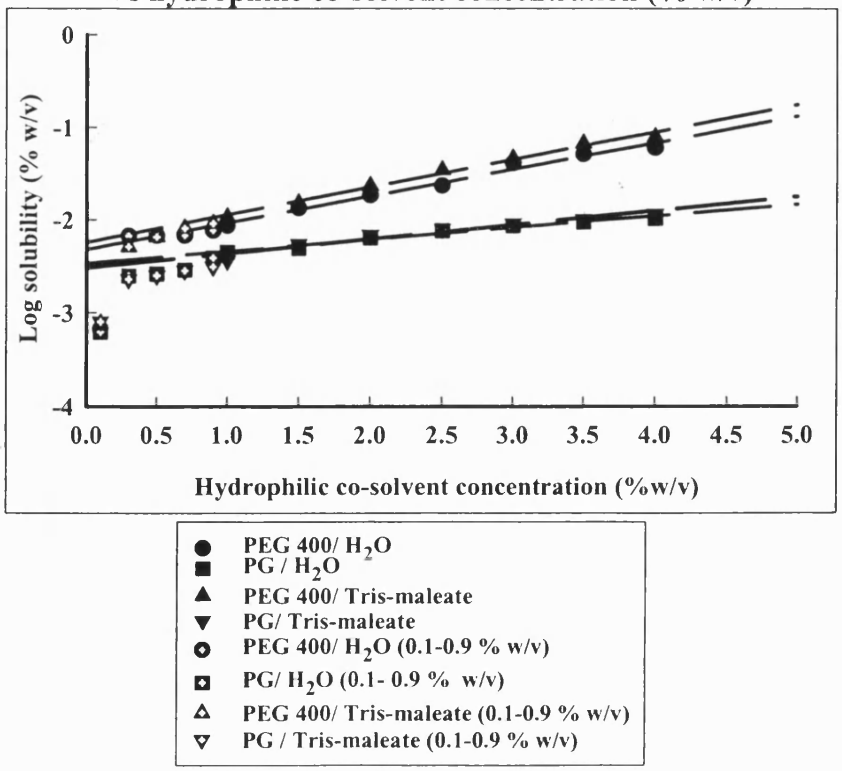
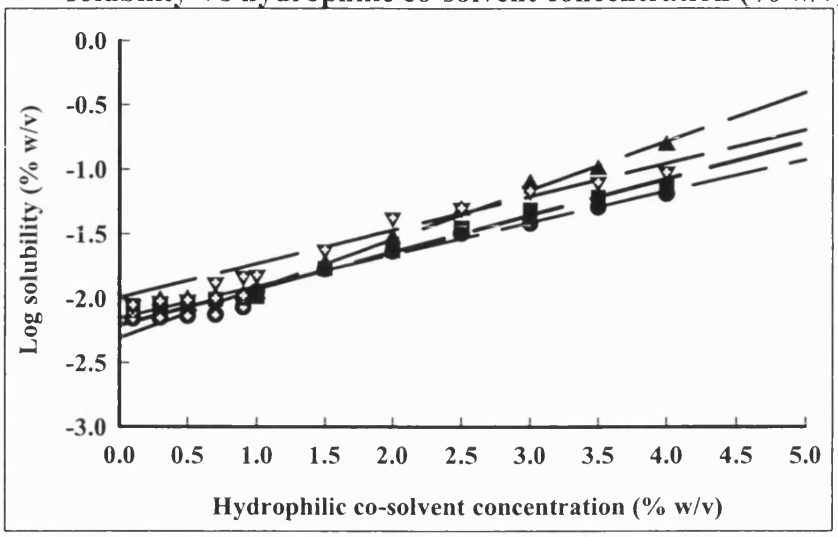


Figure 3.5 The fate of hydrocortisone in water and/or tris-maleate buffer (pH 6.5) of hydrophilic co-solvent at 25°C represented by log solubility Vs hydrophilic co-solvent concentration (% w/v)



- PEG 400/H_2O
- PG/ H_2O
- PG/ Tris-maleate
- PEG 400/ Tris-maleate
- PEG 400/H_2O (0.1-0.9 %w/v)
- PG/ H_2O (0.1-0.9 % w/v)
- PG/ Tris-maleate (0.1-0.9 % w/v)
- PEG 400/ Tris-maleate (0.1-0.9 % w/v)

Figure 3.6 The fate of testosterone in aqueous solution of water and/or tris-maleate buffer pH 6.5 of lipophilic surfactant at 25°C
(the error bars represent the standard deviation of three experiments)

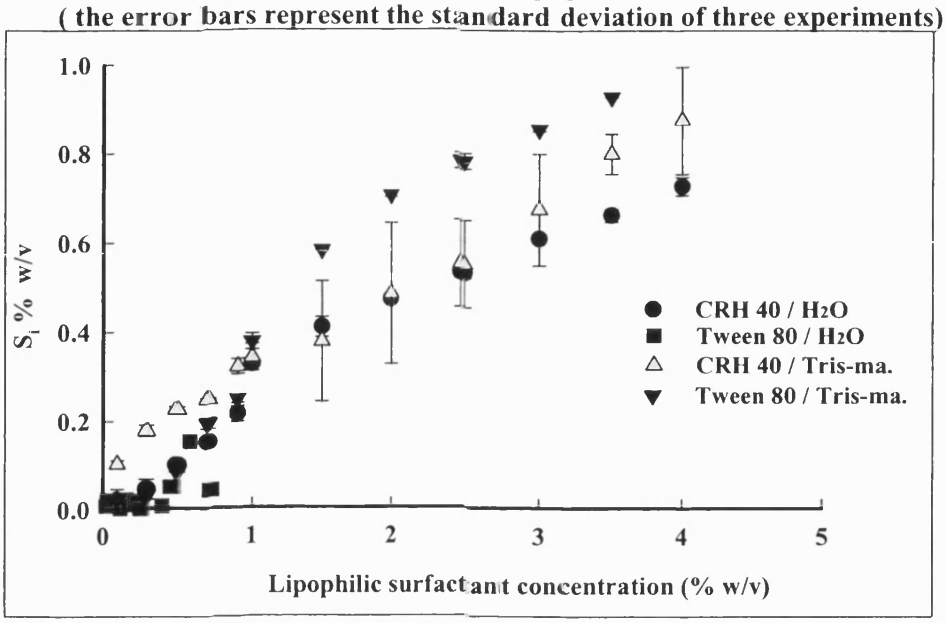


Figure 3.7 The fate of testosterone acetate in aqueous solution of water and/or tris-maleate buffer pH 6.5 of lipophilic surfactant at 25°C
(the error bars represent the standard deviation of three experiments)

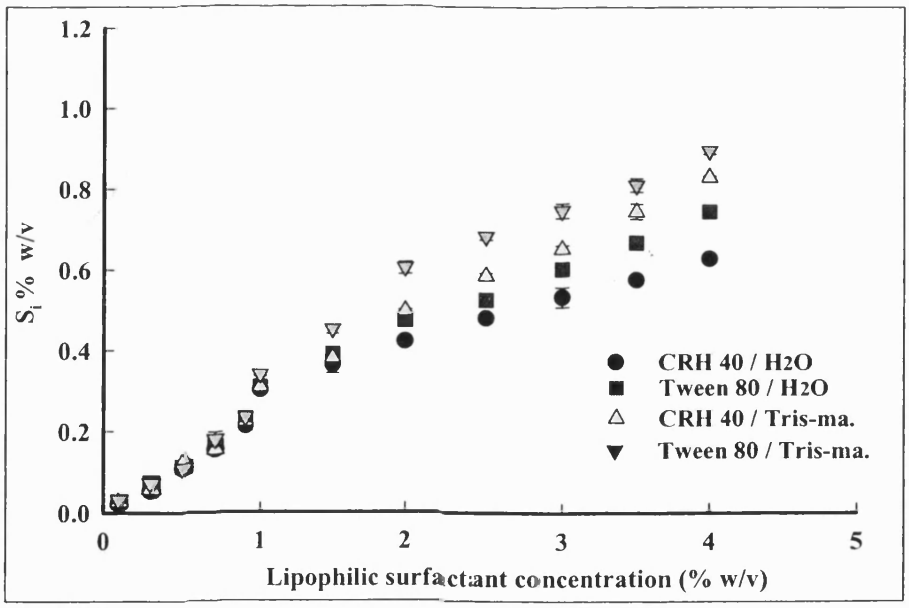


Figure 3.8 The fate of hydrocortisone acetate in water or/and tris-maleate buffer solution pH 6.5 of lipophilic surfactant at 25°C (the error bars represent the standard deviation of three experiments)

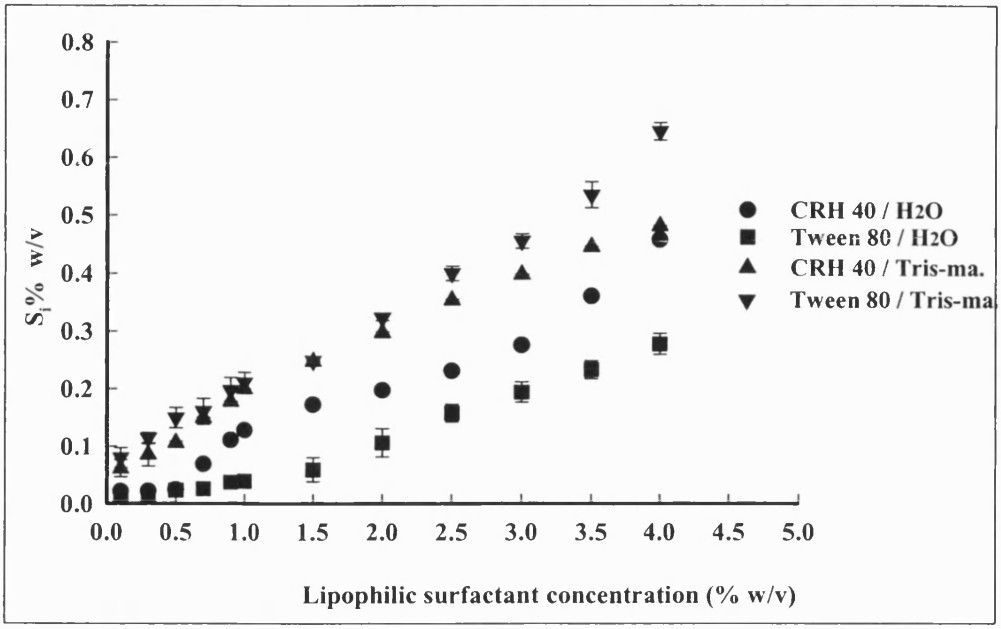


Figure 3.9 The fate of hydrocortisone in water and/or tris-maleate buffer pH 6.5 of lipophilic surfactant at 25°C (the error bars represent the standard deviation of three experiments)

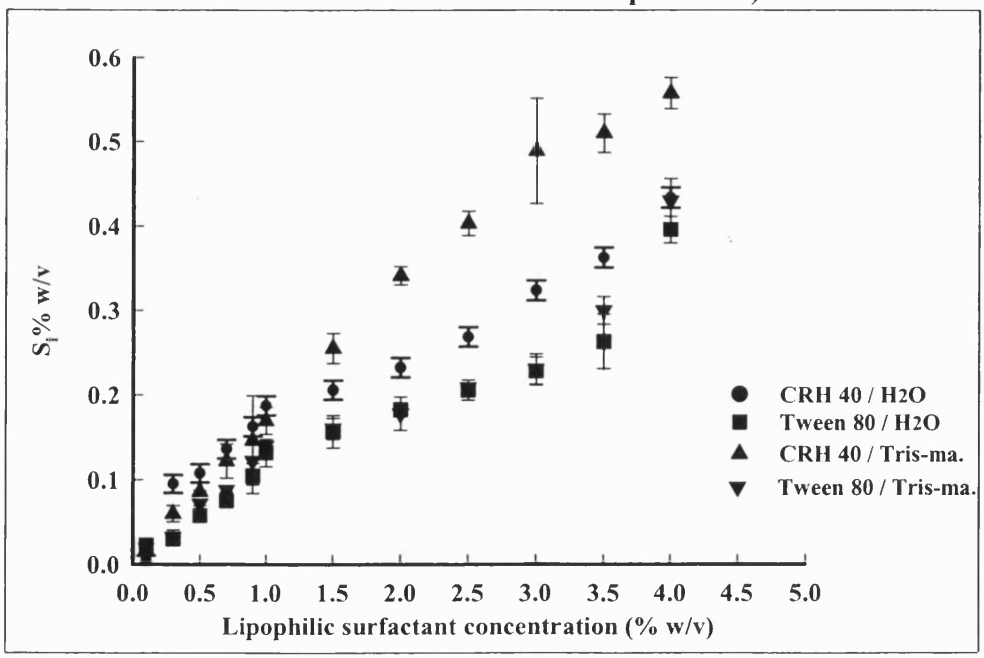
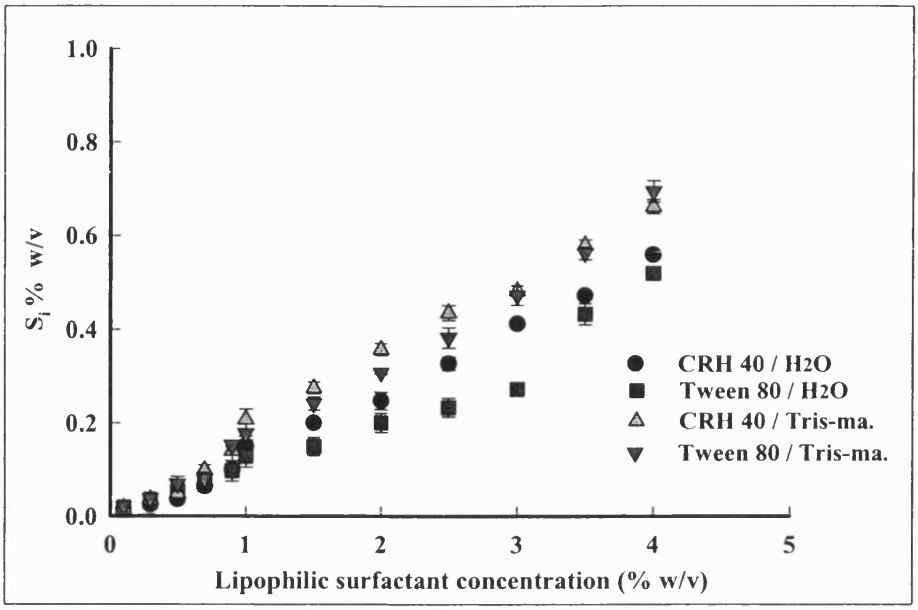


Figure 3.10 The fate of progesterone in aqueous solution of water and/or tris-maleate buffer pH 6.5 of lipophilic surfactant at 25°C (the error bars represent the standard deviation of three experiments)



Chapter 4

In vitro method for simulation of intestinal lipolysis of lipid formulations: the effect of experimental variables on hydrolysis of glycerides using pH-stat method

4.1 Introduction

In order to obtain useful information from an *in vitro* lipolysis model, it is essential to undertake the experiments in a reaction medium that is physiologically representative (MacGregor *et al*, 1997). However, it is complicated by the fact that the GI environment is subject to inter- and intra-individual variations, making it difficult to choose a single set of reaction conditions for in vitro model studies. It is therefore advantageous to determine the effects of key physiologically variables on the rate of lipolysis (Alvarez *et al*, 1989).

A pH-stat is used to monitor the progress of enzymatic hydrolytic reactions where protons are liberated (Brocklehurst, 1992). The use of a pH-stat in many biochemical reactions has been practised for at least 100 years, (Verger *et al*, 1977). A major effort in lipolysis research is directed toward establishing the assay procedure using the pH-stat (Alvarez *et al*, 1989, Challis, 1991, Solomon, 1998). Using the pH-stat method enabled the extent of digestion of a lipid –based formulation, in a physiologically representative reaction medium, to be predicted with respect to time.

The primary objective was to determine the pancreatic lipase-colipase activity present in a crude extract from porcine pancreas [termed "Pancreatin"] and to validate the experimental method. The influence of various physiological factors [e.g. variations in lipase, BS concentrations, BS: LC ratio and variation in pH] on the rate of lipolysis were investigated. A study was also performed to establish the importance of triglyceride chemical structure on the rate of lipolysis via digestion of four different triglyceride oils. Several control experiments were performed to ensure validity of the results obtained using the pH-stat method. The set up, calibration, operation and maintenance of the pH-stat are discussed,

followed by the methodology chosen for the *in vitro* model of lipolysis. This assay procedure was used for all lipolysis experiments described in chapters 5 and 6.

4.2 Principles and theoretical basis of pH-stat assay of lipolysis

4.2.1 Basic principles of the assay

The use of the radiometer pH-stat allows continuous titration, in which the pH is kept approximately constant by frequent addition of an alkali or acid (Dixon *et al*, 1979). The pH-stat operates by monitoring the electrode potential (expressed as pH), during a chemical reaction (Solomon, 1998). The instrument is pre-set to an end-point pH value, which corresponds to the constant pH at which the reaction is required to take place. When the chemical reaction is started, protons are liberated or taken up, with a resultant change in the potential, the autotitration process start, which keeps the pH constant by continuous addition of acid or base. The lipolysis kinetic analysis can be expressed by drawing a progress curve, during the course of a reaction by plotting the volume of titrant as function of time.

4.2.2 The relation of lipase activity to lipolysis assay

Pancreatic lipase-colipase catalyses the lipolysis of the triglycerides by splitting the ester linkages resulting in with one monoglyceride and two fatty acids. The fatty acids release protons with a resultant drop in pH. The pH-stat senses the increased proton activity and titrates a measured volume of NaOH to maintain the initial pH. The number of moles of sodium hydroxide used can be then be equated with moles of fatty acids to calculate the percentage of triglycerides digested through the course of the reaction.

4.2.3 Sources of errors in the lipolysis process

The main difficulty in measuring lipolysis of slowly digesting oils over long periods of time (*e.g.* 1 hour) is that the pH of the reaction medium can fall due to factors other than lipolysis of triglycerides. The liberation of amino acids from

protein degradation (pancreatin is an impure extract and may contain proteolytic enzymes capable of digesting insoluble fragments) and the lipolysis of lecithin are important factors affecting measurement of the lipolysis process. Other problems are the presence of unknown liquid junction potential between saturated KCl and the reaction mixture, and the ability of the liquid from the calomel electrode (situated below the surface of the reaction solution) to leak. The effect of any diffusion potential upon the potential measured by the pH-stat was considered to be minimal and was therefore disregarded.

In the current state of pH-stat measurement, it is necessary to obtain reliable constant stirring (ensuring a reproducible surface interface), and an effective system to prevent absorption of atmospheric carbon dioxide. The control functions that determine the rate of addition of titrant, therefore, are of considerable importance. If settings are too slow, delays may occur in titrant addition whereas rapid addition of titrant can result in overshooting the required end-point pH value. The concentration of titrant, buffer capacity and size of the sample are the factors, which affect selection of an appropriate titration rate. The proportional band selector determines the volume of titrant added and the pause between additions. This selector is set to a pH span prior to the end-point pH value. Titrant delivery is gradually reduced as the end-point pH is approached. The setting of the proportional band selector is normally chosen so that a reduction in titrant delivery starts when nearly 90% of titrant needed.

To compensate for possible interference due to these factors, blank or control experiments were performed to determine the proportional band and titration speed settings. Initial lipolysis experiments were performed in tris-maleate buffer, which has the advantage of avoiding problems caused by other buffers of calcium salt precipitation and absorbance of carbon dioxide. Also, tris-maleate buffer can be used over a wide range (pH 5.5-8.6), which covers the physiological pH range between (4.8-8.2) found in the duodenum (Bowman *et al*, 1967). The appropriate concentration of titrant (sodium hydroxide) was 1 M, ensuring addition of a low volume to the reaction mixture, therefore minimising dilution effects and possible reduction in reaction temperature.

4.2.3.1 Investigation of blank rate.

A blank rate is defined as the existence of an apparent reaction within an assay system without one of the reaction component (Tipton *et al*, 1992). If the blank rate is significant, its source needs to be understood and the rate be quantified so that appropriate corrections can be made. In the standard pH-stat assay, the volume of titrant added should be directly related to release of fatty acid from triglyceride with no interference from a blank rate. A series of control experiments as performed with various components eliminated in turn. A blank rate occurred only when enzyme was present in the system. Adding pancreatin to tris-maleate buffer caused an immediate drop in pH followed by no further release of titrant over 60 min. A 0.02 ml volume of sodium hydroxide (1 M) corrected the drop in pH within 2 sec; this volume was constant for all experiments performed. The drop in pH could be due to acidity of the enzyme suspension or endogenous substrate material contained within it. However, adding pancreatin to simulated bile solution consumed 0.05 ml of sodium hydroxide (1 M) during 60 min, which was slightly higher than the amount added in case of tris-maleate buffer. Verger *et al* (1984) suggested that the presence of lecithin could be responsible for the increase of the amount of sodium hydroxide, because addition of pure pancreatin porcine lipase in the presence bile salt is able to hydrolyse the *sn*-1 ester bond of egg lecithin. It was found that subtraction of the blank rate in the case of kinetic studies is not appropriate because the blank rate obtained is part of integral enzyme activity. Thus to discount it would result in an underestimation of the enzyme activity. In the case of kinetic reactions, subtraction was also considered unnecessary due to minimal and constant nature with the blank rate compared to the overall titrant volume recorded.

4.3 Materials

4.3.1 Tris®-maleate buffer

Trizma®-maleate (tris-maleate)	$C_4H_{11}NO_3C_4H_4O_4$	50 mM	Sigma	T3128
Sodium chloride	NaCL	150 mM	Sigma	S9625
Calcium chloride dihydrate	$CaC_{12}.H_2O$	5 mM	Sigma	C3881

Sodium hydroxide	NaOH	qs	Fisons S/4880/60

4.3.2 Pancreatin Lipase – colipase

Pancreatin (a crude extract from porcine pancreas) was used as a source of pancreatin lipase-colipase activity. This material was supplied by Sigma USP specifications (P1500).

4.3.3 Simulated bile solution

Taurodeoxycholic acid sodium salt	3.0-30mM	Sigma T0875
L-α-phosphatidylcholine	0.7-3mM	Sigma P9671
(60 % pure from fresh frozen egg yolk)		

4.3.4 Glycerides

Tributyrin	$C_{15}H_{26}O_6$	Sigma T8626
Miglyol 812®	(Fractionated coconut oil)	Hüls (UK)
Imwitor 988®		Hüls (UK)
Capmul MCM®		Abetic Corp.
Corn oil		Sigma

4.3.5 Additional materials

Anti-foam A (100 active silicone Polymer)		Sigma A5633
Normadose sodium hydroxide solution	1 M	Prolabo32/066/606
Standard pH 4 phthalate buffer		Fisons J/2820/17
Standard pH 7 phosphate buffer		Fisons J/2850/17
Standard pH 9.2 borate buffer		Fisons J/2870/15

4.4 Equipment

4.4.1 The pH-stat equipment

- Radiometer pH-state system ETS822 was comprised of the following parts:

PHM82 pH meter

TTT80 Titration assembly with stirrer (No.847-714)

ABU80 Autoburette .

Glass indicating electrode (No.GG2040C)

Calomel pH reference electrode (No. K4040C)

*All from Radiometer Analytical A/S, Copenhagen, Denmark.

- Gallenkamp water bath
- B&T Circon pump
- Water-jacketed titration vessel (250ml capacity)
- Magnetic stirrer

The pH-stat was assembled as a single unit. However, the reaction vessel was connected *via* tubing to a pump unit and a heated water bath which circulated the temperature controlled water to the reaction vessel. An integral thermoregulator on the water-bath maintained temperature with a separate thermometer suspended in the water bath to act as an external check (Solomon 1998).

4.4.2 Preparation of buffer solutions

Buffer solutions with pH values in the range 5.0 to 8.5 were prepared by dissolving appropriate quantities of tris-maleate, sodium chloride and calcium chloride dihydrate in distilled water followed by addition of sodium hydroxide pellets to adjust to the required pH at the experimental temperature

Standard buffer is 1 L solution composed of

- 11.86g of tris-maleate
- 0.74g of $CaCl_2.\ 2H_2O$
- 8.77g of NaCl
- 1.59g of NaOH for pH 6.5 or 3.24g of NaOH for pH 8.5 or any other quantity according to the pH required.

4.4.3 Preparation of simulated bile solution (SBS)

The simulated bile solution was prepared daily to minimise degradation of the components. Sodium taurodeoxycholate and phosphatidylcholine (lecithin) were dissolved in tris-maleate buffer of the specified pH (detailed in section 4.4.2) with the aid of heated magnetic stirrer. The heat and agitation were maintained for approximately 30 min at 35 °C, until the lecithin was in solution. The solution had an optically turbid or opalescent appearance. The solution was stored at 4°C until required.

4.4.4 Preparation of pancreatin solutions

The crude extract of pancreatin with distilled water was prepared in an appropriate ratio (*e.g.* 500 mg of pancreatin + 2 ml distilled water for a 250 mg/ml lipase solution) in polyethylene glycol 10 ml tube, followed by mixing for 3 min (using a Whirlimixer from Fisons, Loughborough, UK) to ensure thorough mixing. The lipase solutions, containing insoluble materials, were equilibrated at the required temperature for 20 min prior to use. At this point, the solution was mixed and centrifuged using a Jouan B 3.11 bench top centrifuge (model No. FS200B, Decon laboratories; UK) for 30 min at 3226 g. Then supernatant solution was kept at 4°C. The pancreatin solution was warmed up to 37°C before addition to the reaction. The lipolysis process started the moment the pancreatin solution was added.

4.4.5 Preparation of emulsions

An appropriate amount of triglycerides or SEDDS samples were weighed in a vial and added to reaction medium (100 ml) *i.e.* pH 5.0-8.5 tris-maleate buffer, which was placed in the reaction vessel. The vial used to weigh sample was rinsed three times using remaining simulated bile solution. During the emulsification process foam is formed due to the presence of bile salts and lecithin, and so 10µl Antifoam agent (Sigma, UK) was added. At this point the stirrer was switched on and the medium was left to equilibrate to 37°C (±0.5°C) for 10 min. When a stable pH reading was obtained for 10 min, the pH of the emulsion was raised to

the exact reaction pH (*i.e.* 5.0- 8.5) by addition of sodium hydroxide from the autoburette with a record made of the initial pH and volume of titrant used. The emulsion was left for another 30 min to stabilise at the required pH before starting lipolysis. When a stable pH reading was obtained, the pancreatin solution (section 4.4.4) was added to the reaction medium, which initiated the assay. An electronic timer and the titration were started simultaneously at this stage. The volume of titrant was recorded manually using the timer at intervals of every 10sec for the first twenty min followed by readings every 5 min for a further 40 min

4.4.6 pH-stat conditions

4.4.6.1 Parameters of pH-stat

The following parameters were determined before establishing the assay method:

PHM82

Sensitivity	left as set after calibration (± 1% absolute)
Temperature	37°C or 25 °C
Accuracy	± 0.01 pH at 25 °C

ABU80

Speed	1.25ml/min
Volume	1 ml
Man/auto	auto
Accuracy	imprecision display 0.05 ml
Accuracy in range 0 to 25 ml	titrant volume ±7 µl ± 0.15%

TTT80

Proportional band	0.05 pH units (accuracy ± 5%)
Delay second	pH-stat
UP button	depressed (when end-point pH > reaction medium pH)
End-point accuracy	± 0.02 pH units

4.4.6.2 PHM82 standard pH meter calibration procedure

A daily calibration procedure was necessary to check electrode function and response of the pH meter. The standard buffer solutions used to calibrate the pH meter were chosen according to the end point pH of the assay. Buffer solutions of pH 4 and 7 were used for an end point below 7.0. When the end points above 7 the buffer solutions pH 7 and 9 were used. All calibration procedures were performed at 37°C.

4.4.6.3 Calibration Procedure.

Electrodes were removed from storage solutions, rinsed with distilled water and located in the electrode head. The pH meter sensitivity dial was set at 100 % and the temperature dial to 37°C. The standard pH 7 buffer was left in the reaction vessel until a stable pH reading (± 0.01 pH units) had been displayed for 30 min. Using the buffer dial to alter parallel displacement, the pH reading was adjusted to the manufacturers specified pH value at 37°C unless the correct reading was already shown. The pH was monitored for a further 15 minutes to ensure the reading remained stable. The second buffer solution (pH 4 or 9.2, as appropriate) was placed in the vessel and left until a stable pH reading had been displayed for 15 min. If adjustment to the manufacturers specified value was necessary this was performed using the sensitivity dial, which alters slope. A check was made to ensure the reading remained stable for 15 min. If the pH reading was within ± 0.01 pH units of the manufacturers specified value then calibration was complete. If the pH reading fell outside of these limits the whole procedure was repeated until results were within the specification.

4.4.6.4 Method for standard pH-stat assay

The standard pH-stat assay discussed here was used for all lipolysis experiments; any further modifications made to the assay for a particular investigation are described under the corresponding experiments.

4.4.6.4.1 Preliminary assay procedure

Pancreatin (250mg/ml) was prepared by weighing powder into a 25ml disposable polyethylene glycol plastic tube, and making up to volume. The required amount of triglycerides was weighed into an amber glass vial. If lipolysis of a pure surfactant was under investigation, and the surfactant was solid at room temperature, liquefaction (by heating) was necessary to ease handling and ensure dispersion into the reaction mixture.

4.4.6.4.2 Assay method.

Simulated bile solution (100 ml) was required for each assay; 90% of the solution was placed in the reaction vessel. The previously weighed sample of triglycerides or mixture of triglycerides and surfactant was added. The vial was rinsed using the remaining SBS, followed by 10µl of anti-foam A. The stirrer unit was activated immediately; the reaction medium was left to equilibrate to 37°C (+0.5°C) for 10 min. Then the mixture was titrated up to the required pH end-point, with a record made of the initial pH and titrant volume used. This step allowed adjustment for slight variations in starting pH values of the reaction mixture caused by different batches of simulated bile solution and properties of the triglyceride and surfactants (Solomon, 1998). While the reaction medium was reaching equilibrium temperature, a vial of pancreatin solution was heated at 37°C for a pre-incubation period of 20 min. Addition of 1ml stock pancreatin solution initiated the reaction. The titrator and the electronic timer were switched on at this stage. The volume of titrant used was recorded manually using the timer at intervals of 10 sec for the first 20 min followed readings every 5 min for a further 40 min.

4.4.6.4.3 Clean up and switch off procedure

After each experiment, the vessel was washed using thoroughly soap and distilled water, followed using ethanol 99%v/v to ensure no contamination occurred between assays. Electrodes were cleaned by wiping that with clinical tissues, followed by distilled water rinse, and a final wipe with absolute ethanol 99 %v/v.

The calomel pH reference electrode was placed in a solution of saturated potassium chloride while the glass electrode was placed in standard pH 4 buffer solutions.

4.4.6.4.4 Assessment of the pH-stat results

The assay gave readings of the volume of titrant used per unit time, which corresponded to protons released from fatty acid on a one-to-one molar basis. In the literature, the workers assume each triglyceride molecule underwent hydrolysis to form two fatty acids and a monoglyceride molecule. All free fatty acids were ionised; therefore, it was possible to determine the percentage of triglyceride digested. For tributyrin, the calculation was based on complete hydrolysis to three fatty acids and glycerol. Hydrolysis of short-chain fatty acids (SCT) (tributyrin) to completion was assumed to be rapidly cleaved, especially *in vitro* in the presence of bile salts (Gurr *et al*, 1971). The percentage of triglyceride digested out of the total initially present was then determined from the concentration of free fatty acids. The percentage of triglyceride digested or amount of fatty acid neutralised was plotted against time to produce a progress curve of lipase activity referred to in future text as a digestion profile.

4.5 Expression of lipase activity of pancreatin enzyme.

Pancreatin was used a source of pancreatin lipase-colipase activity. It is of interest that the relative amounts of hydrophobic amino acids in the pancreatic lipases from human sources do not differ from those found for other pancreatic enzymes (Verger *et al*, 1973; Borgström *et al*, 1977b). Porcine pancreatin enzyme contains a single peptide chain with an NH_2-terminal serine and COOH-terminal cystine, and has a high degree of sequence similarity to human derived source with a similar pH-activity profile (Vandermeers *et al*, 1974; Breg *et al*, 1995). Lipase has been defined as an interfacial enzyme that specifically interacts with water-insoluble substrate (Borgström *et al*, 1977b). Bile salts inhibit lipase by preventing the binding of the enzyme to its substrate; colipase binds a bile-salt covered triglycerides-water interface and provides a high affinity "anchor" site for lipase (Borgström *et al*, 1977).

Tributyrin is the substrate preferred over long chain triglycerides to determine the activity of pancreatin for three reasons (I) the product formed, butanoic acid, is completely ionised to water-soluble product under alkaline conditions (pH 8 to pH 9), (II) there is no need to add any emulsifier because it emulsifies spontaneously in dilute sodium chloride solution (Borgström *et al*, 1984), (III) tributyrin is hydrolysed between two and ten times faster than long chain triglycerides. This results in a linear reaction rate, which is normally maintained for a sufficient time period to assess maximum activity.

Two batches of crude pancreatin were used in this project; the supplier specified a minimum lipase activity corresponding to the United States Pharmacopoeia (USP) specification. Since pancreatin lipase was prepared as a crude extract, lipase activity was determined to ensure reproducibility between batches I and II of pancreatin used throughout this study.

4.5.1 Method

Pancreatic lipase activity was measured with a pH-stat titrator by continuously titrating the released free fatty acids with 1 M NaOH at pH 8.5 at 25°C. The tributyrin emulsion was prepared from mixing tributyrin 2.4 g in 100 ml buffer containing 50 mM tris-maleate pH 8.5, 5 mM calcium chloride dihydrate and 150 mM sodium chloride. The pancreatin suspension was prepared in distilled water (150 mg/3 ml), mixed by vortexing and incubated at 25°C followed by one minute thorough mixing before use. For each assay, tributyrin emulsion 100 ml and 25 mg of pancreatin (0.5 ml of suspension) (section 4.4.4) were used. The emulsion was left to equilibrate in the reaction vessel for 30 min with continuous stirring before the addition of enzyme. Lipolysis was initiated by the addition of pancreatin solution to triglycerides. An electronic timer and the pH-stat were activated immediately. The initial pH, pH 8.5, was maintained by continuously titrating the liberated fatty acids with 1.0 M NaOH. The amount of lipolysis was noted every 10 sec for 20 min. The highest volume of titrant used, over six consecutive readings, corresponded to the maximum catalytic potential of the enzyme per min under assay conditions.

4.5.2 Results

The activity of pancreatic lipase is normally expressed in terms of tributyrin units or TBUs (Borgström *et al*, 1984). One TBU is the enzyme activity that can liberate 1mmole of fatty acid per minute from tributyrin at 25°C and pH 8.5 in the presence of 5 mM $CaCl_2$ and 150 mM NaCl. Normal human duodenal contents are reported to contain 1000 TBU of colipase-lipase per ml. The enzyme activity of pancreatin (TBU per mg of pancreatin) was used to express the activity of the enzyme, as shown in Table 4.1.

Batch No.	Pancreatin Supplier	Batch No.	Specific activity of pancreatin (Maximum No. of TBU per mg per minute).
I	Sigma	28H0518	6.0
II	Sigma	99H11617	5.6

Table **4.1** Lipase activities determined for each batch of pancreatin used in the standard pH-stat assay.

From Table 4.1, it can be seen that there is a minimal difference between batches in terms of TBU per mg of pancreatin. To assess the reproducibility of kinetics results generated from the experimental model, the activities of pancreatin from three separated lipolysis experiments for each pancreatin batch performed under identical reaction conditions were compared. The mean values of TBU mg min were obtained for each batch represented in the Table 4.1. The coefficient variation of batches, I and II was 4.3% and 3.9% respectively. The experimental model indicated highly reproducible results.

4.6 Enzyme concentration

For most enzymes acting independently in solution, the initial velocity (V) of the reaction would be expected to be directly proportional to the concentration (E) of the enzyme (*i.e.* zero activity at zero enzyme concentration).

$$V=K(E)$$

This is the normal case, but deviations can occur, either due to an artefact of assay system or of the enzyme itself (Dixon *et al*, 1979). The initial velocity relationship to enzyme concentration was measured to ensure that no interference was present from the standard pH-stat assay system. Further, experiments were performed to determine the initial velocity of enzyme after centrifugation. This approach enabled determination of the enzyme concentration (250 mg of crude pancreatin) selected for use in the standard pH-stat assay throughout this work.

4.6.1 Method

The digestion profiles of SCT (TBU), MCT(Miglyol 812®) and LCT (Corn oil®) were assessed over a 1 hour period using three different enzyme concentration (500 mg/ml, 250 mg/ml, 50 mg/ml), prepared as described in section 4.4.4. As a quantity of 250 mg/ml pancreatin had been proposed for use in the rest of experiments, a series of lipase solutions were prepared (250mg/ml) from pancreatin by mixing the crude extract with distilled water and/or with tri-maleate buffer, as mentioned in section 4.4.4. The resulting solutions, which contained insoluble material, were then held for different times of centrifugation (10, 20, 30, 60, 120) minutes. A series of digestion profiles were performed for both pancreatin batches I and II.

4.6.2 Results

The lipolysis of SCT, MCT, and LCT was assessed over a 1 hour period using three different concentrations of enzyme. A linear relationship between initial velocity and pancreatin concentration (r^2=0.9876) was obtained, if data 500 mg for pancreatin were omitted. Frequently, the linear portion of an assay is sufficiently prolonged to the initial rate. It can be estimated accurately simply by drawing a tangent or by calculating taking first derivatives of the early part of the progress curve, where loss of linearity occur relatively rapidly due to depletion of substrate or reading equilibrium. Decreasing the enzyme concentration to slow down the rate of product formation, and increasing the sensitivity of the assay method, may prolong the linearity. It has been commonly assumed that measurement of reaction rates should be restricted to a period in which 5 % or less

of the substrate has been utilised (Segel, 1975). The rate of lipase activity follows a direct but non-linear, correlation. For example, Figure 4.1 showed that increasing the concentration of pancreatin from 50 mg/ml to 250 mg/ml promoted the percentage lipolysis of TBU after 20 min by a approximately 3.2 fold. In contrast, when the concentration of pancreatin lipase increased from 250 mg/ml to 500 mg/ml, the rate of lipolysis after 20 min was raised by 1.2 fold, as shown in Figure 4.1. The previous observation was observed to MCT (Miglyol 812®) and LCT (Corn oil®) as shown in Figures 4.2 and 4.3 respectively.

When considering the suitability of using 250 mg/ml of pancreatin in the standard pH-stat assay, different samples from batch II and I were centrifuged for (10, 20, 30, 60 &120 min) at the same speed 3226 g. The initial velocities decreased with increasing time of centrifugation until a plateau was recorded at 3226 g for 30 min as presented in Figure 4.4. Therefore, 250 mg/ml of pancreatin centrifuged for 30 min was concluded to be appropriate for use in the standard pH-stat assay. The digestion profile of SCT produced maximum extent of digestion possible at pH 8.5 at 25°C as shown in Figure 4.5. Figures 4.5 and 4.6 showed that the effect of reconstitution of pancreatin batch I with tris- maleate buffer and/or water. The resulting initial velocity of the enzyme suggests a higher activity towards the substrate of SCT in tris-maleate buffer than in water. Therefore, following to this result, the enzyme was reconstituted using the tris-maleate buffer throughout this research.

4.7 Selection of the triglyceride substrate

The method of choice for selection of the appropriate triglycerides suitable for use in the standard pH-stat assay was susceptibility to the hydrolytic activity of pancreatin. Five triglyceride substrates were analysed under conditions of the standard pH-stat assay for of 60 min (section 4.4.5), to ensure that any inhibition of the apparent enzyme activity due to the presence of another substrate could be easily detected.

4.7.1 Results

The percentage of total triglyceride after 60 min is summarized in Table 4.2 for each triglyceride. The various triglycerides were analysed at equal weight (mass) approximately 1g under the same condition of the standard pH-stat (pH 7.0, 37°C) assay, therefore use of a percentage calculation eliminated errors introduced by their different molecular weight values and hence molar concentration. Each sample was reanalysed three times taking the average and the coefficient of variation. They calculated based 2FA released from TG and 1FA from DG. From the data shown in Table 4.2, although MCT, fractionated coconut oil was chosen for further investigation because of the high total percentage of digestion in the assay system. It is pharmaceutically acceptable, has a high standard of purity, low cost and availability with specified proportions of fatty acids. Other glycerides were used for further investigation that helps to clarify the effect of different parameters of the pH-stat assay system.

4.8 Effect of pH on enzyme activity

In general, enzymes are only active over a limited range of pH and in most cases, an optimum pH is observed (Dixon *et al*, 1979). The pH optimum for porcine lipase with tributyrin as substrate is in the range 8-9 (Borgström *et al*, 1973). The optimum pH for lipase activity will depend upon conditions of the assay medium, such as the concentration of bile salt, the ionic strength of the buffer and temperature (Borgström, 1954). The fasting duodenal pH in healthy subjects is 6.5, so the standard pH-stat assay was run at pH 6.5 in all-previous work (Borgström *et al*, 1984). Therefore, the standard pH used through the research is pH 7.0. The maximum velocity of pancreatin activity towards different type of substrates was therefore assessed over a pH range of 5 to 8.5.

4.8.1 Method

All lipolysis profiles were performed under standard pH-stat assay conditions, using five different substrates (section 4.7) at approximately1 g. End-point pH values were used between 5.0 and 8.5 at increments of 0.5. Simulated bile

solution for each experiment was prepared using tris-maleate of an apparent pH value. A concentrated solution of tris-maleate buffer was prepared without sodium hydroxide. The buffer solution was then adjusted to the required pH by adding solid sodium hydroxide and was made up to the volume required. Simulated bile solution was equilibrated at 37°C and the pH was adjusted with 1 M sodium hydroxide, if required.

4.8.2 Results

A series of digestion profiles SCT, LCT and MCT (expressed as millimoles of fatty acids liberated with time using a range of pH between 5-8.5), in triplicate for each end-point pH are shown in Figures 4.7-4.9. The initial velocity of the reaction was determined after ensuring no lag phase was present at the start of the reaction. In Figures 4.7-4.9 showed that a lag phase occurred at high pH 8 and 8.5, while at pH 7.5-6.5 typical hyperbolic curve was obtained. The percentage digestion of different SCT, LCT, mixed mono-, di- and tri-glycerides and MCT after 20 min and 60 min varies with pH as shown in Figures 4.1-4.14 respectively. For tributyrin at pH 7.5, 60.8% of the total triglycerides had been digested after 60 min. Some triglycerides appear to have undergone hydrolysis approximately half the amount of fatty acids and glycerol present, *e.g.* MCT (Miglyol 812®) the percentage of digested triglycerides was 49.05% at pH 7.5. For LCT (Corn oil®) at pH 6.5, 10.5% of the total triglycerides present had been digested, whereas at pH 7.5 this figure increased to 29.67 %. This may be due to a high rate of enzyme activity coupled with a high degree of emulsification conferred by the presence of bile salt. The bell-shaped graph of the pH-rate profile as shown in Figures 4.10-4.14 could be considered as the composite of two sigmoidal titration curves. The ionisation status of the ionic groups present in the enzyme or substrate may explain the observed decrease in enzyme activity away from the optimum pH 7.5 (Segel, 1975). The free enzyme, the enzyme-substrate complex or the substrate may undergo such catalytic changes. It is known that the N-terminal domain of the pancreatic lipase contains the catalytic triad Ser, His, Asp and is responsible for triglyceride hydrolysis (Hermoso *et al*, 1996).

Trade name	Scientific name	Dominant fatty acids	Molecular weight	% purity	% of TG digested	Manufacture
Tributyrin	Tributyrin(SCT)	$C_{4:0}$	302.4	99%	65% (CV=0.807%)	Sigma, UK
Imwitor 988®	Mixture of caprylic glycerides	DG = 46.1 % MG = 53.9 %	271.92	99%	59.30% (CV=4.6%)	Huls Gmbh.
Capmul MCM®	Mixture of caprylic and capric fatty acids	TG = 15 % DG = 55 % MG = 30%	330.8	99%	51.16% (CV=1.06)	Abetic Corp.
Miglyol 812®	Mixed decanoyl C8:0and Qctanoyl glycerides C10: (MCT)	$C_{6:0}$ (max.6%), $C_{8:0}$ (50-65%), $C_{10:0}$ (25-35%), $C_{12:0}$ (max.2%)	520	99%	49.05% (CV=0.109)	Huls Gmbh.
Corn Oil®	LCT	$C_{20:1}$ (0.2%) $C_{20:0}$ (0.3%) $C_{18:3}$ (1.6%) $C_{18:2}$ (50%) $C_{18:1}$ (30%) $C_{18:0}$ (2.3%) $C_{16:1}$ (0.3%) $C_{16:0}$ (14%) $C_{14:0}$ (0.6%)	866.6	99%	23.5% (CV= 0.912)	Sigma, UK

Table 4.2 Percentage of digestion of triglycerides under standard pH- stat conditions

Semeriva *et al* (1971) suggested that the histidine of pancreatic lipase needs to be ionised to achieve the maximal rate of hydrolysis of tributyrin. Early work used a pK_a value for histidine in their system of 5.8 although the environment of the enzyme can influence the exact pK_a values (Borgström, 1958). An effect of pH on the stability of the enzyme, which may become irreversibly inactivated, is known to occur with porcine pancreatin lipase at pH values lower than 5 (Verger *et al*, 1984). Borgström *et al* (1977b) indicated that the rate-limiting step of lipolysis at alkaline pH is the slow formation of ternary lipase-colipase-bile salt complex and its adsorption to the substrate interface. The effect may occur in combination; for instance, the fall on both sides from the optimum may be due either to a fall in affinity due to a decreased saturation of the enzyme with substrate and /or to instability of the enzyme. This could be confirmed by further experimentation involving pre-incubation of the enzyme for a period equal to the length of the assay at various pH values before commencing digestion at pH 7.5. If the reaction rate was decreased, this would indicate pH-related enzyme inactivation. The emphasis in this work is characteristics assay conditions rather than the enzyme activity. The choice of the buffer and the ionic strength of the simulated bile solution are important factors and need further consideration. When making up a buffer with a given molarity at different pH values, the ionic strength will vary with pH. Tris-maleate has been used to cover a wide range of pH from 5.8-8.6 and exhibits the smallest changes in ionic strength, because the conjugate base has a charge (Z)=0 (Stevens *et al*, 1972). If necessary, solutions could have been brought to constant ionic strength by adding the appropriate quantity of a neutral non-inhibitory salt.

4.9 Effect of bile components

The two major solutes of bile are bile salts and lecithin. Bile salts are not only water-soluble end products of cholesterol metabolism, but are also amphipathic molecules with multiple physiological functions (Hofmann *et al*, 1992). Bile salts are important surfactants in the biological systems; they solubilize lecithin and cholesterol to form mixed micelles. Bile regulates a number of cholesterol

metabolising enzymes in the liver and intestine and facilitates absorption of dietary fats by emulsifying large droplets, making them more accessible to pancreatic lipase. Further, bile salts remove the products of pancreatic hydrolysis, *e.g.* fatty acids, through micelle formation (O'Connor *et al*, 1984). Bile salts and lecithin are the two major components in bile and the molar ratio is approximately 2:1 to 5:1. Together they are essential to the formation of mixed micelles (Naylor, 1993). As an amphiphilic compound, lecithin exerts a synergistic effect with bile salts *via* the formation of mixed micelles. The final effect on lipolysis depends on the ratio of lecithin to bile salts. Low ratios enhance enzyme activity, whereas high ratios lead to inhibition (Lykidis *et al*, 1997).

Small (1967) demonstrated that the micelle actually expands or swells with an increase in lecithin content. This facilitates the uptake of biological lipids such as cholesterol, fatty acids, steroids, and fat-soluble vitamins that have very poor solubility when only bile salts are present. In accordance with Brockerhoff (1971), the presence of bile salts keeps the reaction rate constant for a longer period. He interrupted this effect as a protection of the lipase from inactivation by unfolding at the hydrophobic surface of the substrate by increasing the substrate surface area, either by increasing the amount of the substrate or its degree of dispersion; higher bile salts concentrations are needed to prevent inhibition (Borgström, 1993). However, it is difficult to establish from the literature exactly what is the typical level of BS in the human small intestine. Hernell *et al* (1990) has reported that BS concentration is ranging from 5.8-37 mM with an average value of 14.5 mM, ± 8.8 mM. The purpose of the work described in this section was two fold. The initial objective was to determine the ratio of BS to LC and their impact on the lipolysis profile for different substrates. The other objective was centred on determining the effect of bile salt concentration on the rate of lipolysis for different triglycerides.

4.9.1 Method

A series of lipolysis experiments was carried out at 37°C using 250 mg/ml pancreatin lipase (section 4.4.4) and 100 ml of 1% w/v emulsion in standard buffer pH 7.0, prepared using different concentrations of Ox-bile (0, 4, 15, 30, 60,

90, 120, 150, 200, and 250 mM) without using lecithin. In other experiments, three ratios of BS to LC (2:1, 4:1, and 5:1) were added to the standard buffer at a different pH range between 6.0 - 7.5 in 0.5 increments. The ratio of 2:1 and 4:1 were chosen according to Dressman *et al* (1998) fast state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) state. 5:1 ratio normally used by the early workers (Challis, 1990; Solomon, 1998).

4.9.2 Results

It has been common practice to use single synthetic BS NaTDC, rather than any natural BS or a physiologically representative mixture of synthetic BS (Alvarez *et al*, 1989). It has been found that the identity of BS had no appreciable impact on the rate of lipolysis; therefore, Ox-bile has been used instead of NaTDC to determine the effect of bile salt concentration. Moreover, the cost of Ox-bile is less than NaTDC. It was evident that the increase of BS concentration increases the lipolysis rate up to its CMC. The percentages digestion of five different substrate of SCT, MCT, LCT and mixed glycerides are shown in Figures 4.15, 4.16, 4.17, 4.18 and 4.19 respectively. MCT and SCT (Miglyol 812® and Tributyrin) as shown in Figures 4.15 and 4.16 respectively the highest percentage of digestion for the first 20 min was 43.69 % and 70.83 % respectively at 30 mM Ox-bile. In contrast, increasing the Ox-bile to 260 mM followed a decrease after the first 20 min up to 35.23 % and 56.52 % respectively. Figure 4.17 represented that LCT (Corn oil®), when the bile salt concentration increased from 30 mM to 260 mM the extent of lipolysis increased in absolute terms from 36.96 % to 41.96 %. Figures 4.18 and 4.19 showed that mixtures of mono-, di- and tri-glycerides (Capmul MCM®, and Imwitor 988®) followed the same trend as MCT and SCT. When experiments are performed *in vitro* under conditions in which the molar ratio of lipolytic products to BS exceeds 1:1 the rate of lipolysis may be impeded. The experimental system contained 1 g of either SCT (Tributyrin) or MCT (Miglyol 812®) and LCT (Corn oil®). Complete lipolysis of these oils to MG+2FA respectively, yields 5.7 and 3.4 mmole of lipolytic product, given that the reactions were carried out in a volume of 100 ml. These quantities correspond to concentrations of 57 mM and 34 mM.

Clearly, these levels are within the solubilized capacity of BS when the latter are present at 60 mM. As a result, the lipolysis of both MCT and SCT progressed rapidly when BS concentration was 26 mM. Thus, in the case of MCT, a plateau was reached after 20 min, which is approximately 45.78%. It is interesting to speculate why some approximately <4% additional lipolysis was observed in the subsequent 40 min. One possible reason for this was that the amount of BS decreased which facilitated solubilization of more diglycerides. Since pancreatic lipase must bind to an interface to adopt its most catalytically active confirmations; the latter (diglyceride) would there be a less effective substrate for further digestion. Lipase is thus protected from irreversible inactivation, whilst its catalytic activity against the substrate is lost although colipase has a negative charge in the pH range of lipase activity. The effect of bile salts in displacing lipase from the substrate interface parallels the loss of lipase catalytic activity. The inactivation of lipase by bile salt is related to its critical micellar concentration (CMC) and is due to the accumulation of bile salts at the interface, thus giving it a negative charge. Colipase binding is decreased by an increase by bile salt concentration and the extent of binding of colipase to the substrate interface (Borgström *et al*, 1984).

Clearly, experiments *in vitro* with high concentrations of BS will not provide a 'sink' for which lipolytic products can be discharged. The relationship between bile salt concentration and substrate surface area is directly proportional. Lipolytic product removal is a rate-limiting factor for the progress of lipolysis as shown in Figures 4.15 and 4.16. In general, increasing bile salt concentration is dependent on the substrate concentration *i.e.* increasing BS concentration does not necessarily increase the rate of lipolysis because removal of the lipolytic product, is the rate-limiting step.

The ratio of BS:LC was chosen according to Dressman *et al* (1998). The FeSSIF (fed) state contained NaTDC to LC 15 mM: 3.75 mM respectively, while the FaSSIF (fast) state was 3.0 mM:0.75 mM (2:1). A series of lipolysis experiments were run using these ratios and those represented by Solomon (1998) (4:1) 8 mM: 1.5 mM as shown in Figures 4.20 - 4.24. Figures 4.20 and 4.21 respectively

showed that altering the concentration of bile salts and lecithin present in simulated bile solution at pH 7.0 does not alter the extent of SCT and MCT triglycerides for the first 20 min. However, there is an increase in the percentage of digestion of total diglycerides after 60 min. Similar conditions were applied for a mixture of glycerides as shown in Figures 4.23 and 4.24. While for LCT (corn oil) there is a sharp increase in the total digestion of triglycerides after 20 min and 60 min accompanied with increasing of BS to LC 5: 1 as shown in Figure 4.22. Changing the ratio of BS to LC was studied at different pH. The greatest change in the percentage digestion of triglycerides in SCT, MCT and LCT occurred at pH 7.0 as shown in Figures 4.20-4.22 respectively.

4.10 Effect of calcium concentration

Alavrez and Stella (1989) reported that calcium ions play a major role on the rate of triglycerides hydrolysis. Although it has been reported that lipase cofactor does not need ions. Calcium ions have been shown to be involved in the mechanism of action of pancreatic lipase (Borgström, 1980; Armand *et al*, 1992). Calcium ions can modulate either lipase velocity or the lag phase until the maximal enzyme activity is reached. Calcium ions may decrease the electrostatic repulsion occurring at the emulsion-water interface. Excess calcium can induce coalescence of emulsified particles. The calcium concentration required to activate lipase is somewhat higher than that expected to be found in the intestinal contents. The average of calcium concentration in the lumen appears to be diet-dependent and is proposed to be approximates 7 mM (Mansbach *et al*, 1975). However, if the diet does not provide the calcium concentration required to activate lipase, the bile particularly in the gallbladder, is rich in calcium (about 11-38 mM) and can provide a microenvironment necessary for lipase activity. The role of calcium ions is still unclear and hence the effect of calcium ion concentration on different substrates under standard pH-stat conditions was studied.

4.10.1 Methods

Lipolysis experiments were carried out at 37°C using 250 mg/ml pancreatin lipase (6 TBU /mg) (section 4.4.4) and 100 ml of 1% w/v emulsion (SCT, MCT, LCT and mixed mono-,di-, and tri-glycerides) in standard buffer pH 7.0, with BS:LC ratio 4:1 (15mM: 3.75mM) and the calcium concentration was 0 mM, 5 mM and 40 mM respectively.

4.10.2 Results and Discussion

Calcium concentration had a significant effect on the rate of lipolysis approximately 2 fold increase of LCTs (Corn oil®) as shown in Figures 4.25 and 4.26. Based on Borgström (1980) results, in the absence of Ca^{2+} the lag phase was indefinitely long; increases in Ca^{2+} concentration decreased the lag time, but not in the final steady-state rate, as seen with LCT (Corn oil®) as shown in Figure 4.25. The aqueous solubility of the lipolytic product of MCT and LCT is low 10^{-4} and 10^{-7}M, respectively. The lag phase is believed to be measure of the ease of penetration of the lipase-colipase complex into the interface of the TG. The presence of calcium is proposed to be more important for the lag phase than the presence of phospholipids and bile salts. In the presence of calcium, a lipase complex formed with the TDC-phospholipid mixed micelle appears to be the catalytically most effective enzyme complex (Alvarez *et al*, 1989). Such complexation forms a liquid crystalline phase (Patton *et al*, 1979), in which FA are soluble and probably transported to the gastrointestinal membrane to facilitate absorption. Figure 4.26 showed that SCT (TB) was insensitive to calcium concentration, and MCT (Miglyol 812®) were much less dependent than LCT. Mixed glycerides (Capmul MCM® and Imwitor 988®) showed slight increases in the rate of lipolysis proportional to calcium concentration, due to the side chain of triglycerides present.

4.11 Conclusion

In the previous studies, an *in-vitro* lipid digestion model has been established to provide, a detailed and quantity map of the chemical kinetics and phase behaviour of common lipid excipients during digestion, under simulated physiological conditions. Standard pH-stat conditions involve assembling 100ml of aqueous solution of electrolytes, bile salts, solubilized phospholipid, which includes approximately 1g of pure lipid excipients, dispersed using stirring system. The standard pH-stat solution contained 50mM tris-maleate buffer [pH 7.0], 5mM $CaCL_2.2H_2O$, 150mM NaCl, 15mM Na TDC, 3.5mM LC [FeSSIF state]. This is maintained at the desired temperature using a water bath or jacketed vessel. Pancreatin (containing pancreatic lipase and colipase) is added as a concentrated solution (usually 250 mg in 1ml at 6 TBU/ mg). The data showed that the rate and extent of lipid excipients digestion profiles is markedly dependent on the fatty acid chain length. Thus, the rate and extent of medium chain lipid digestion and mixed mono-di-, and tri-glycerides was significantly higher than the corresponding long chain lipid, and the extent of medium chain lipid digestion was effectively independent of bile salt concentration and calcium concentration. Conversely, efficient digestion and dispersion of long chain lipids was much more dependent on bile salt concentration and calcium concentration.

Figure 4.1 Dissociation profile of pancreatin using SCT (Tributyrin) as a substrate using pH- stat method

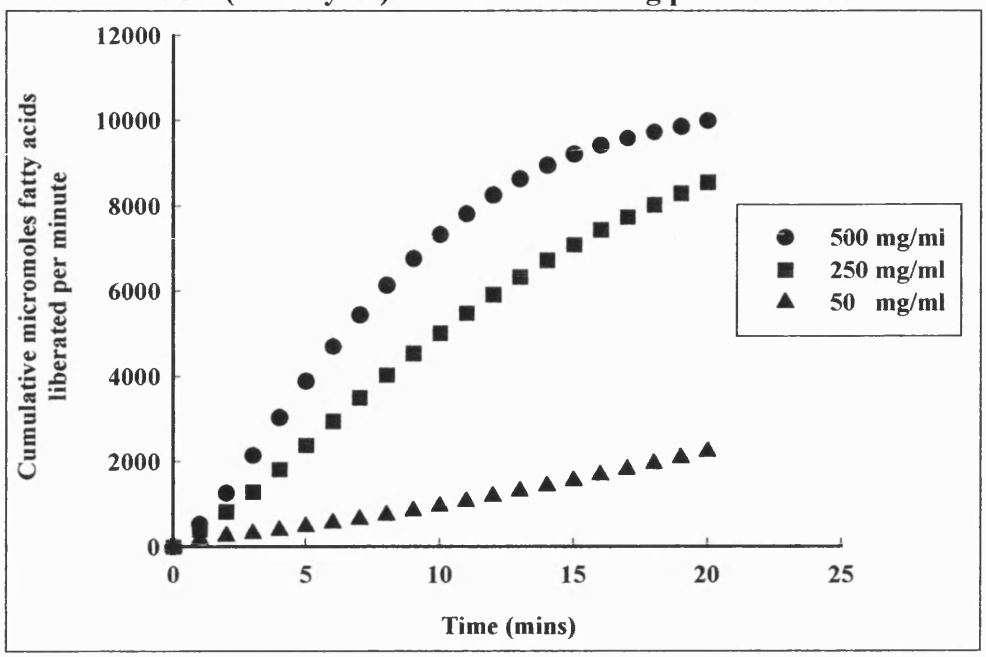


Figure 4.2 Dissociation profile of pancreatin using MCT (Miglyol 812) as a substrate using pH-stat method

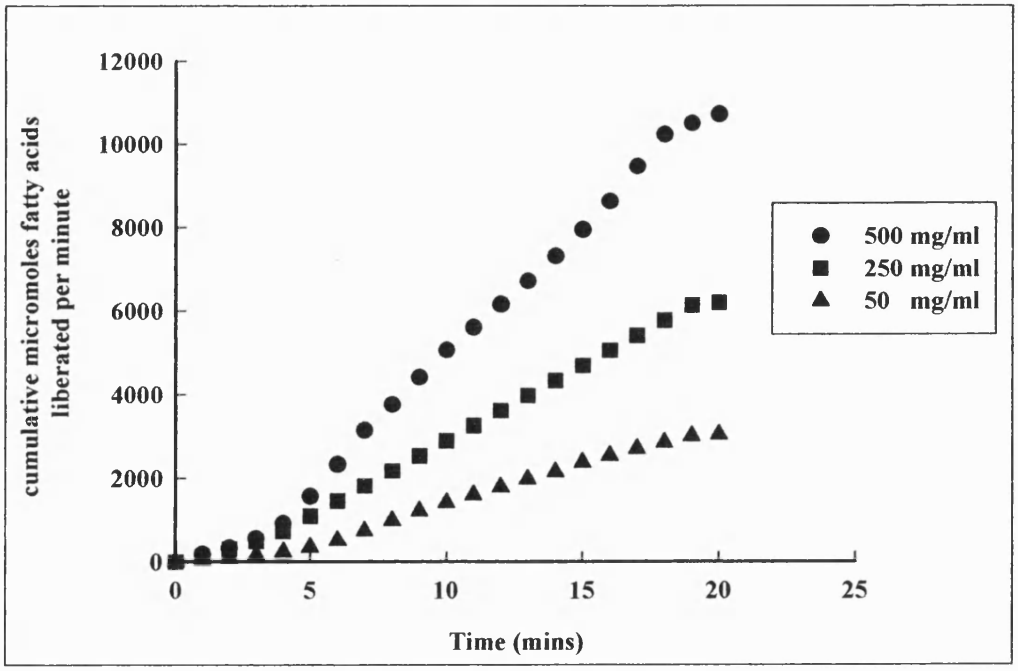


Figure 4.3 Dissociation profile of pancreatin using LCT (Corn oil) as a substrate using pH- stat method

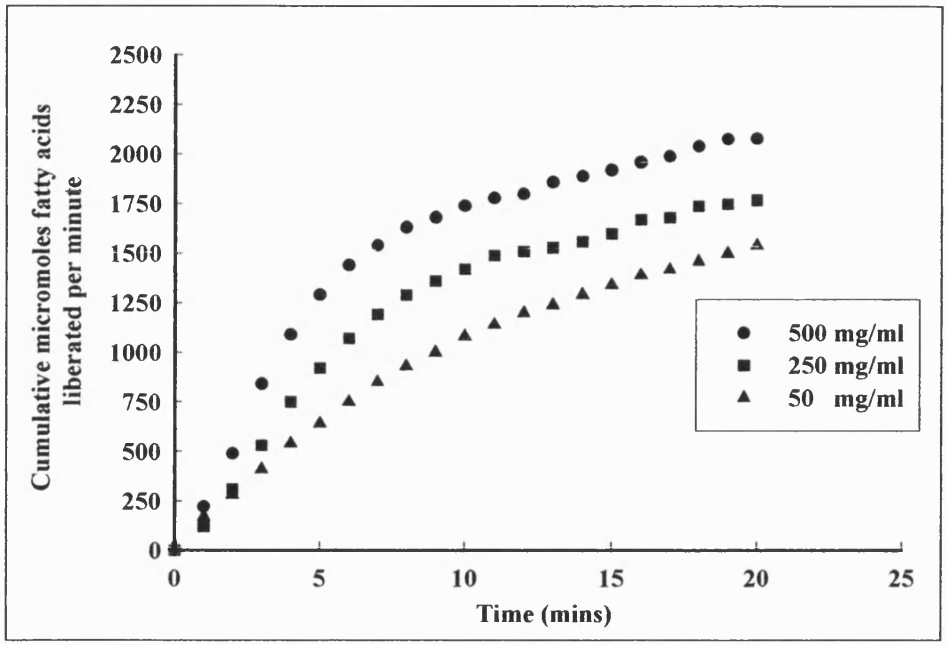


Figure 4.4 Effect of centrifugation time (3226g) on pancreatin activity (How many TBUs per min)using pH-stat method

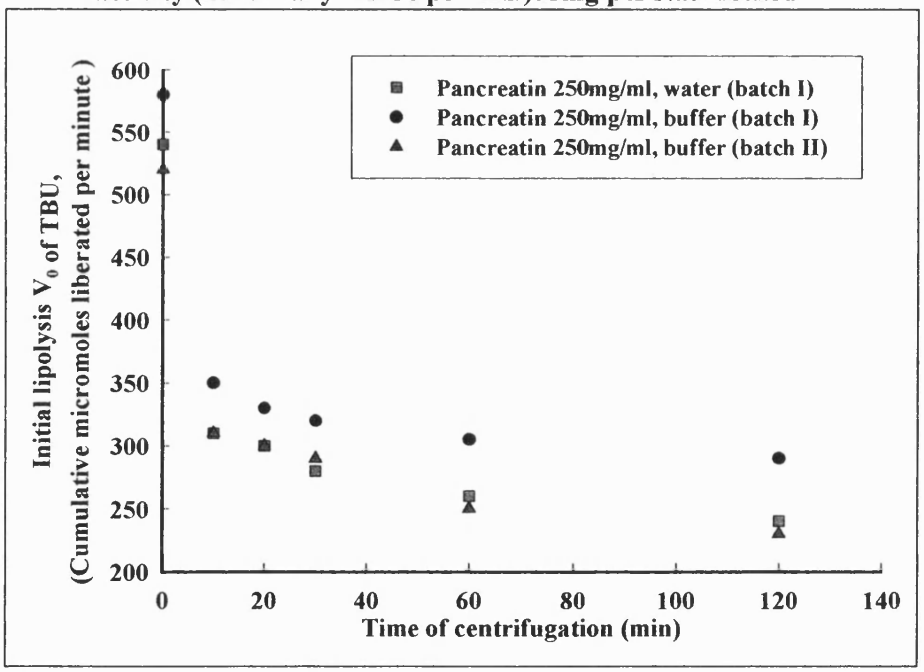


Figure 4.5 Effect of centrifugation time on the dissociation profile of tributyrin in water using 250 mg/ml (batch # 1) using pH -stat method

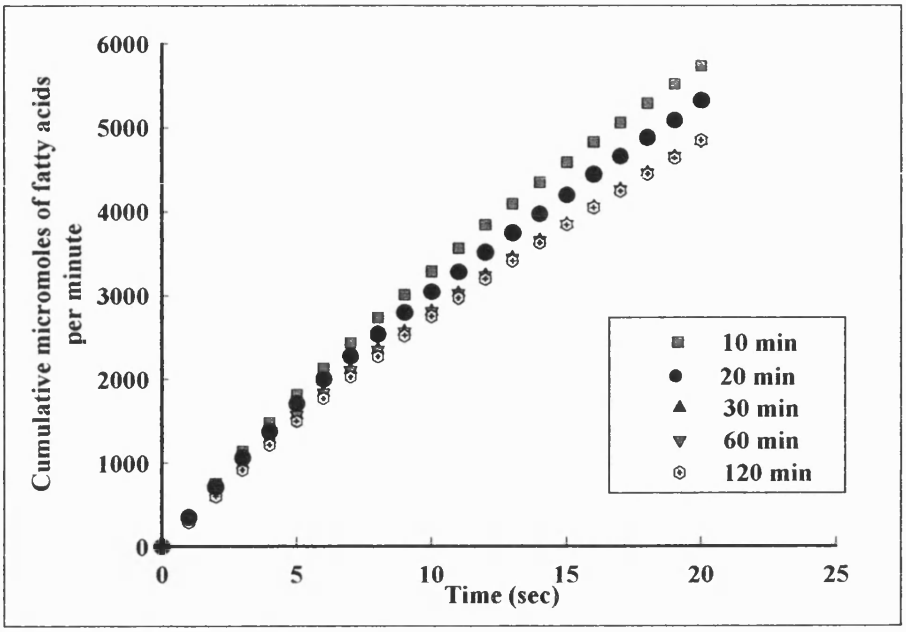


Figure 4.6 Effect of centrifugation time on the dissociation profile of tributyrin in tris-maleate buffer (pH 7.0) using 250 mg/ml (batch # 1) using pH-stat method

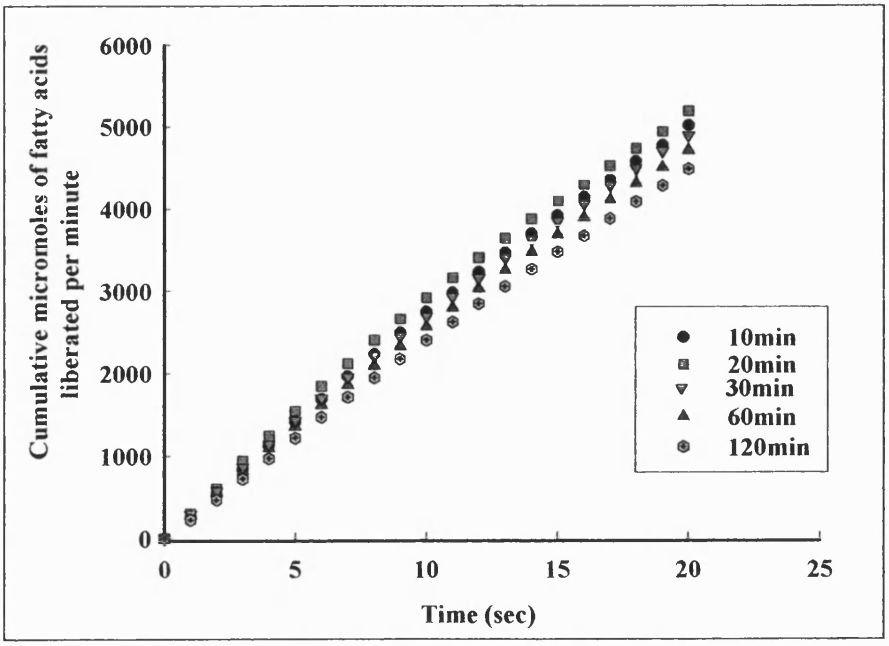


Figure 4.7 Effect of pH on the digestion profile of SCT (Tributyrin) using pH-stat method

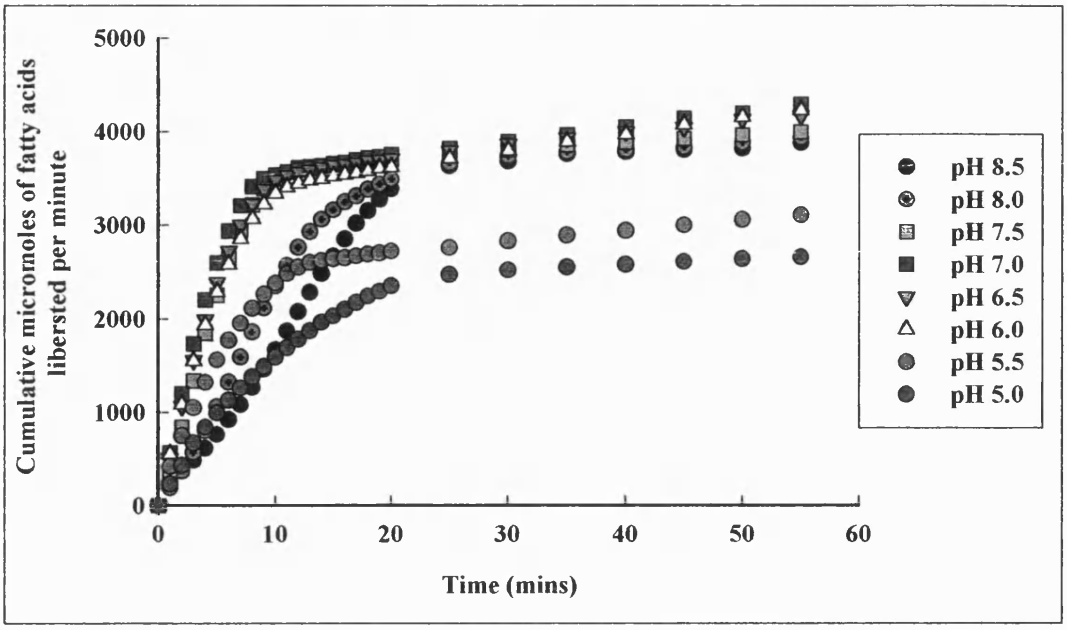


Figure 4.8 Effect of pH on digestion profile of LCT (Corn oil) using pH-stat method

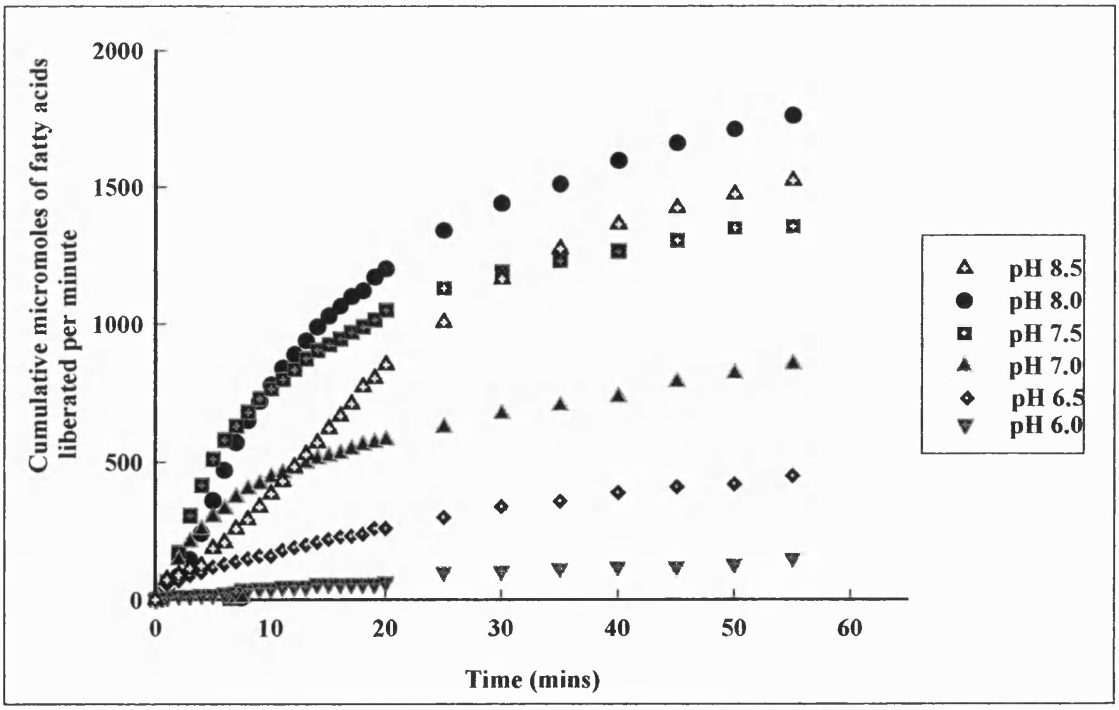


Figure 4.9 Effect of pH range on the digestion profile of MCT (Miglyol 812) using pH- stat method

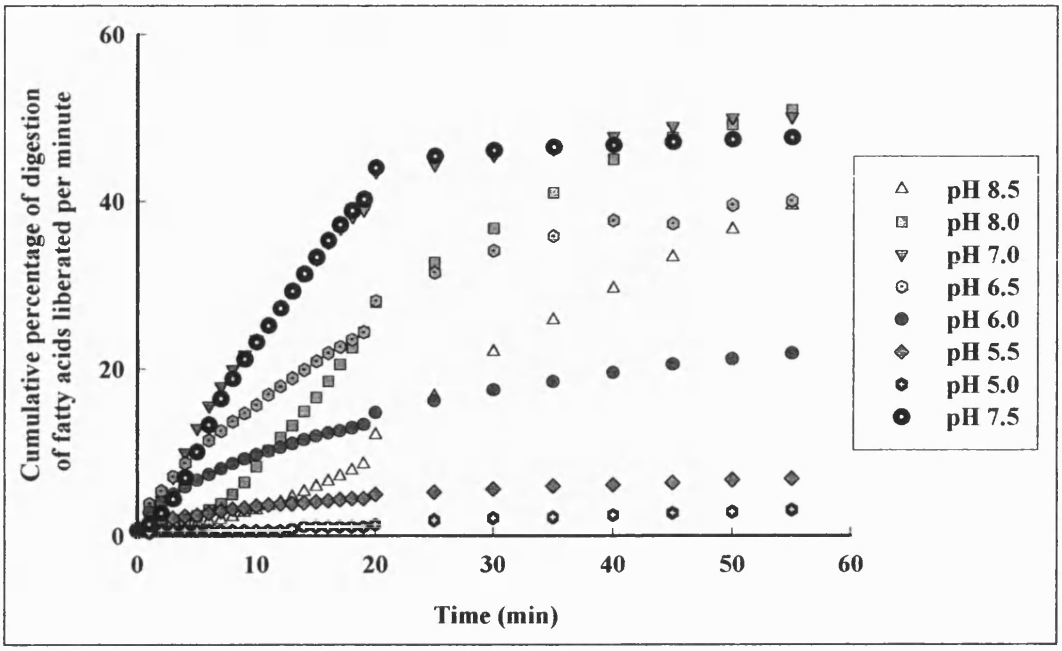


Figure 4.10 The effect of pH on the total percentage of digestion of SCT (tributyrin) using the pH-stat method

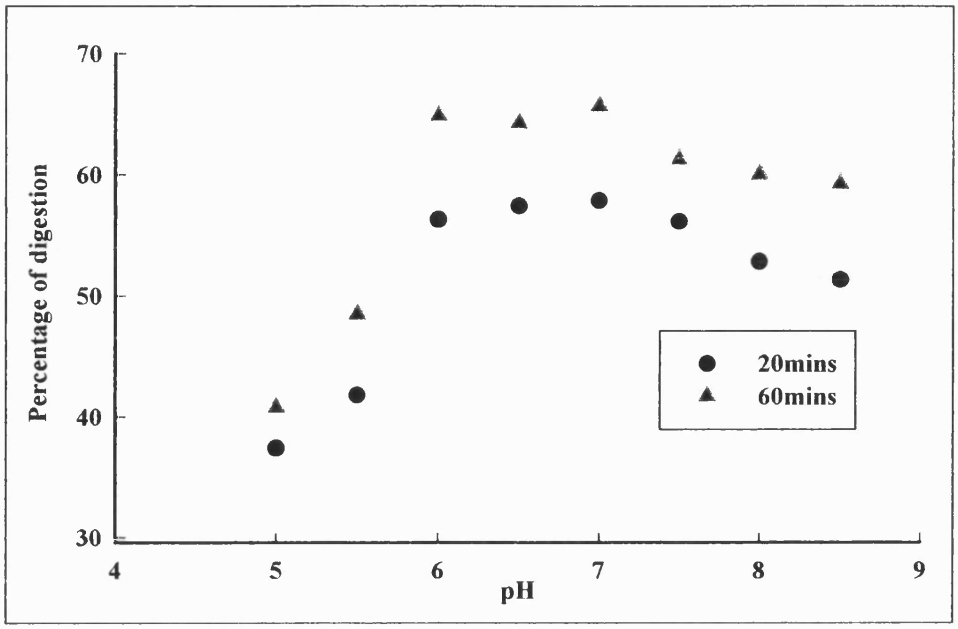


Figure 4.11 The effect of pH on the total percentage of digestion of MCT (Miglyol 812) using the pH-stat method

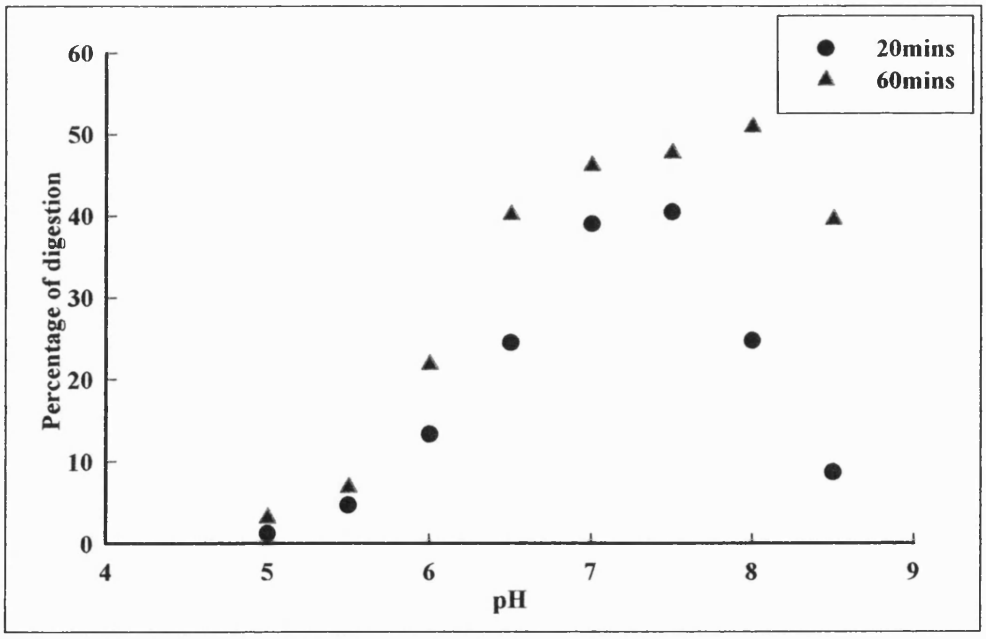


Figure 4.12 The effect of pH on the total percentage of digestion of mixed glycerides mono-, di-, and tri-glycerides (Imwitor 988) using the pH-stat method

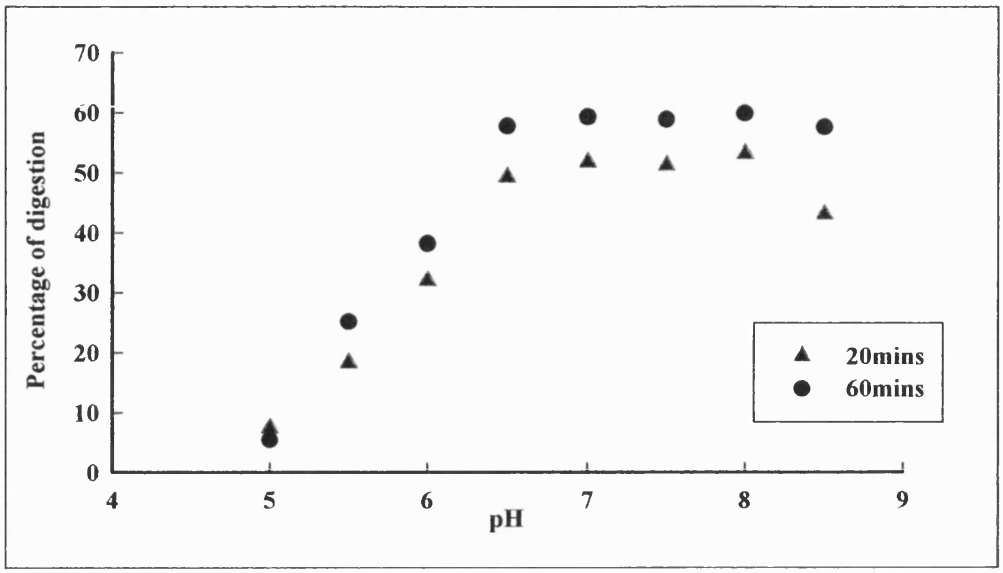


Figure 4.13 The effect of pH on the total percentage of digestion of mixed glycerides mono-, di-, and tri-glycerides (Capmul MCM) using the pH-stat method

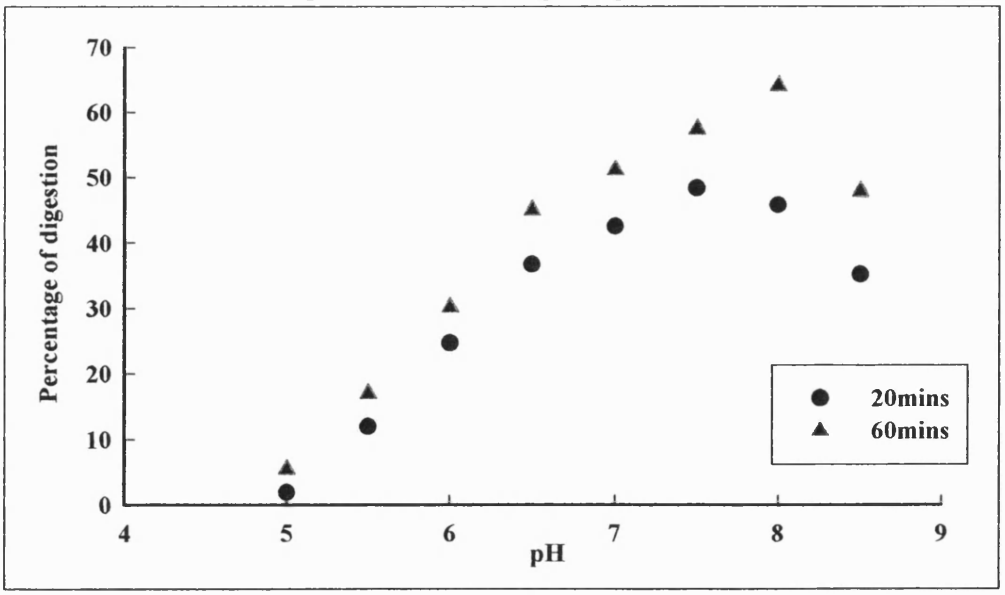


Figure 4.14 The effect of pH on the total percentage of digestion of LCT (Corn oil) using the pH-stat method

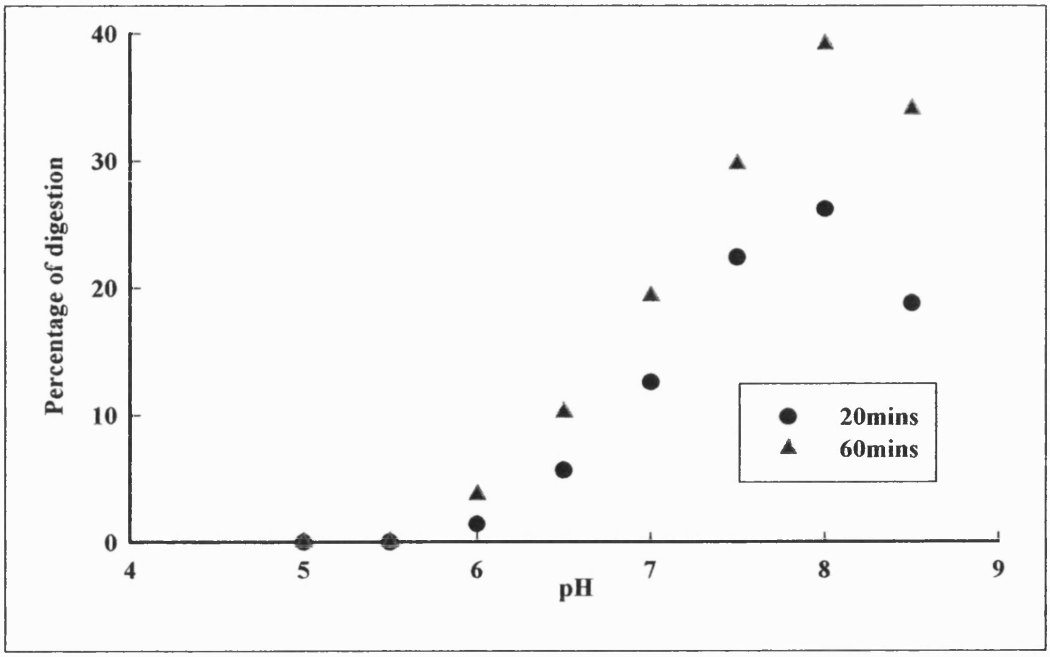


Figure 4.15 Effect of Ox- bile concentration on the total digestion of SCT(Tributyrin) using pH- stat conditions

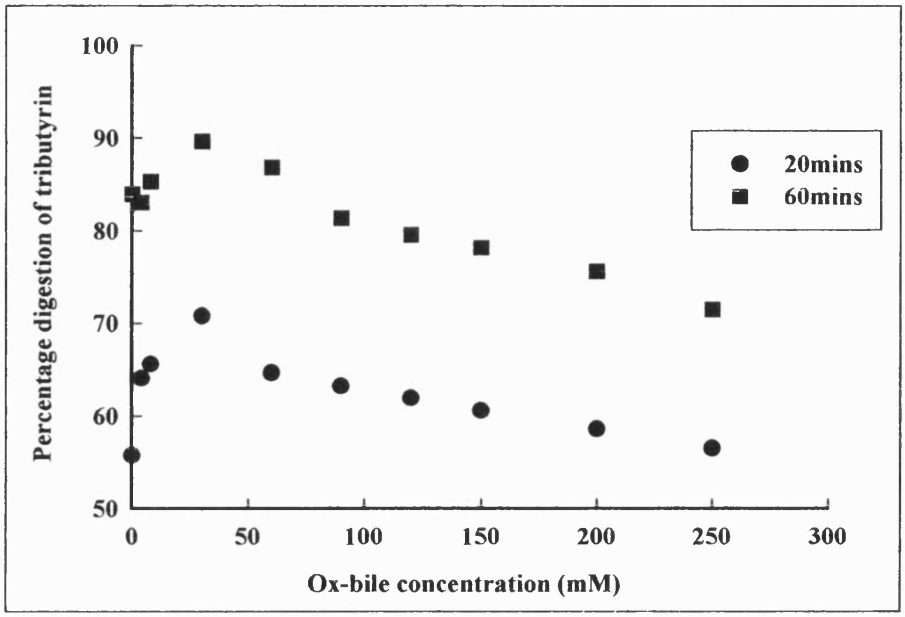


Figure 4.16 Effect of Ox -bile concentration on the total digestion of MCT (Miglyol 812) under standard pH-stat conditions

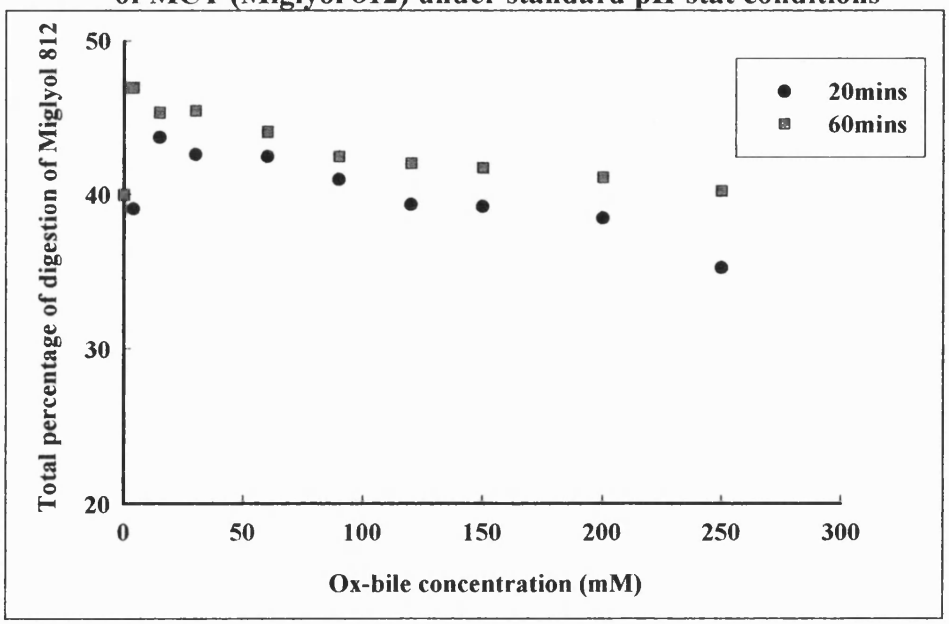


Figure 4.17 Effect of Ox-bile concentration on the total digestion of LCT (Corn oil) under standard pH-stat conditons

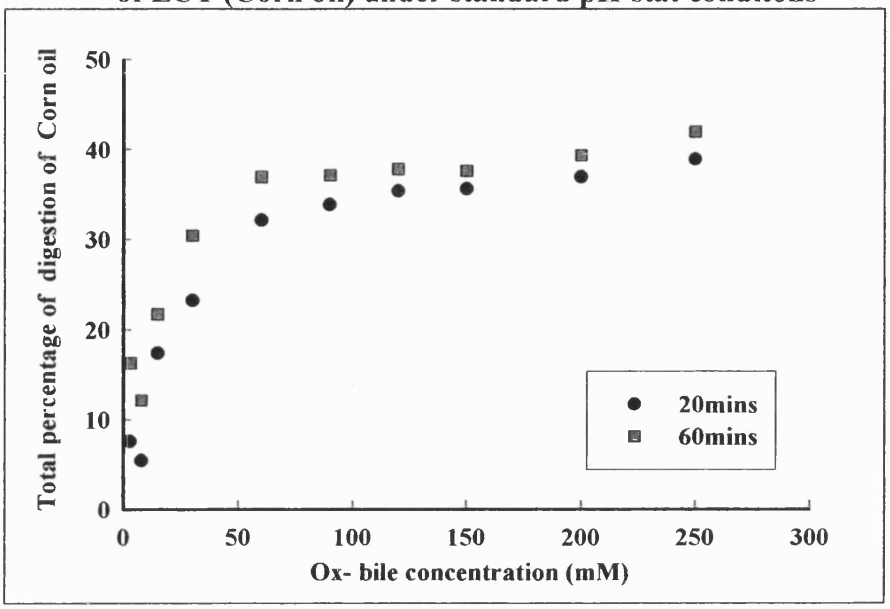


Figure 4.18 Effect of Ox-bile concentration on the total digestion of mixed mono-, di- and tri-glycerides (Imwitor 988) under standard pH-stat conditions

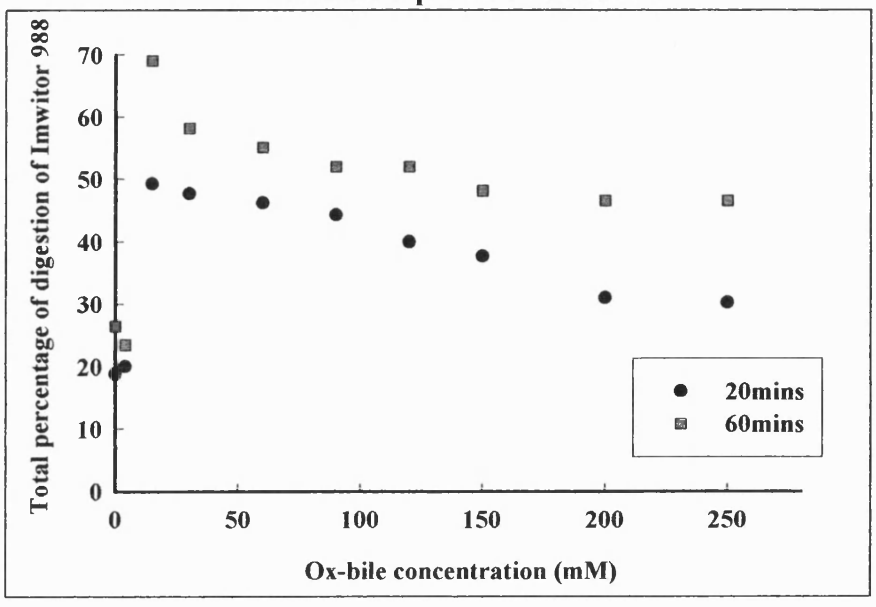


Figure 4.19 Effect of Ox-bile concentration on the total digestion of mixed mono-, di- and tri-glycerides (Capmul MCM) under standard pH-stat conditions

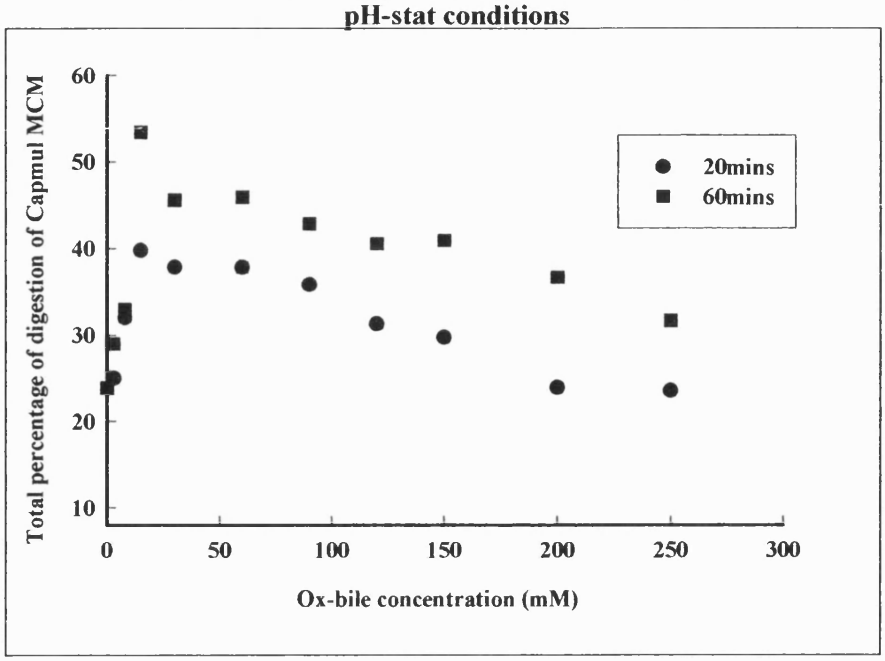


Figure 4.20 Effect of BS : LC ratios on the total percentage digestion of tributyrin under standard pH-stat conditions

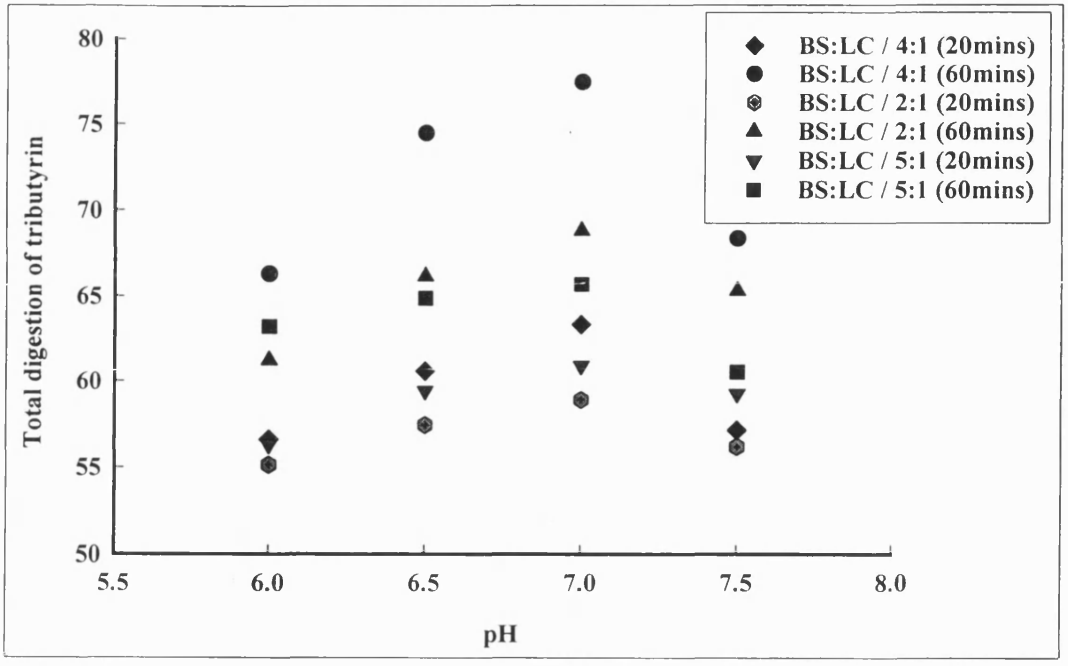


Figure 4.21 Effect of BS:LC ratios on the total digestion of MCT (Miglyol 812) under standard pH-stat conditions

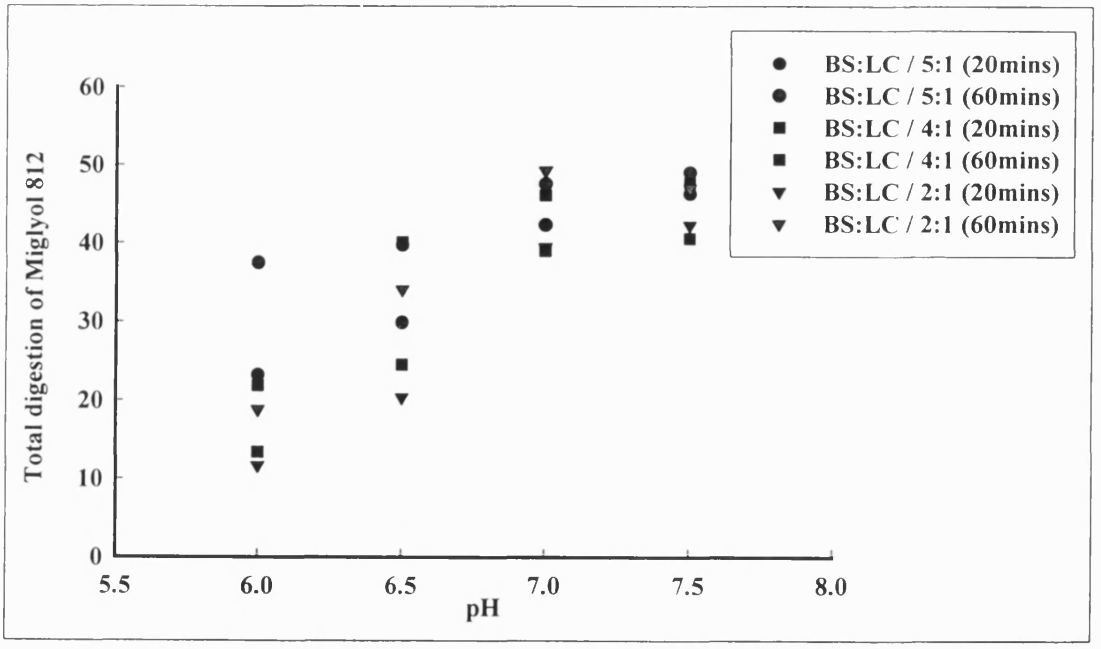


Figure 4.22 Effect of BS:LC ratios on the total digestion of LCT (Corn oil) under standard pH-stat conditions

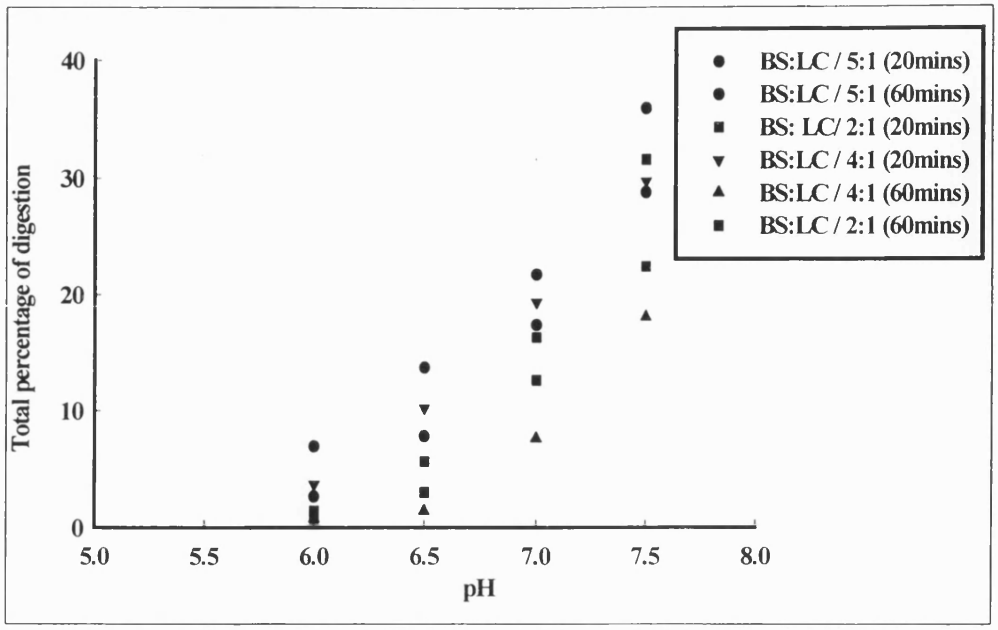


Figure 4.23 Effect of BS:LC ratios at different pH on the total of digestion of mixed glycerides mono-, di-, and tri-glycerides (Capmul MCM)

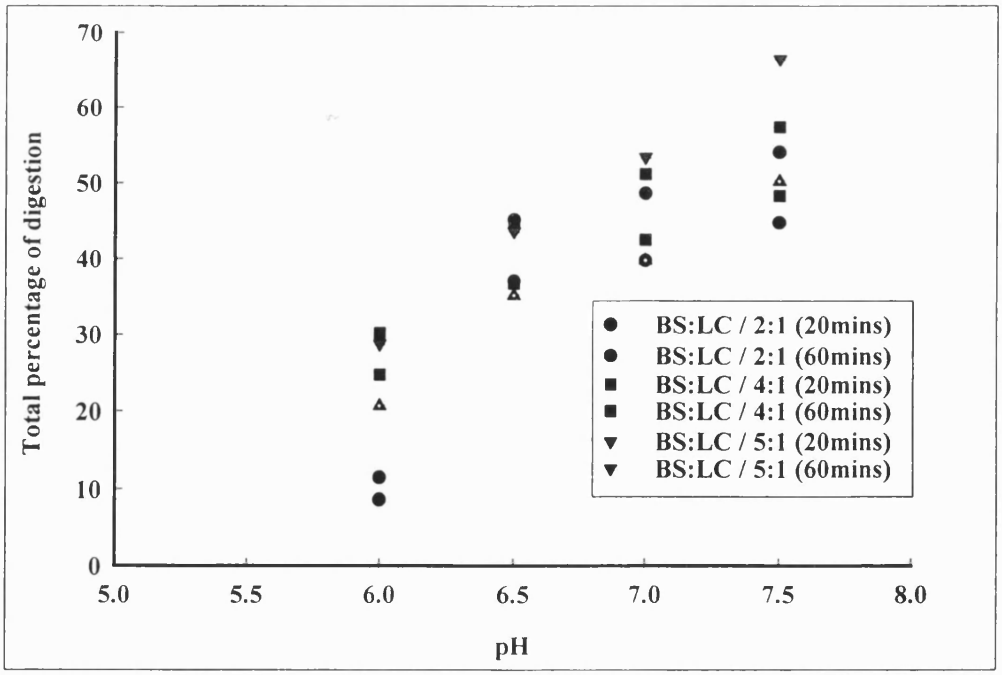


Figure 4.24 Effect of BS : LC ratios on the total digestion of mixed mono-, di- and tri-glycerides (Imwitor 988) under standard pH-stat conditions

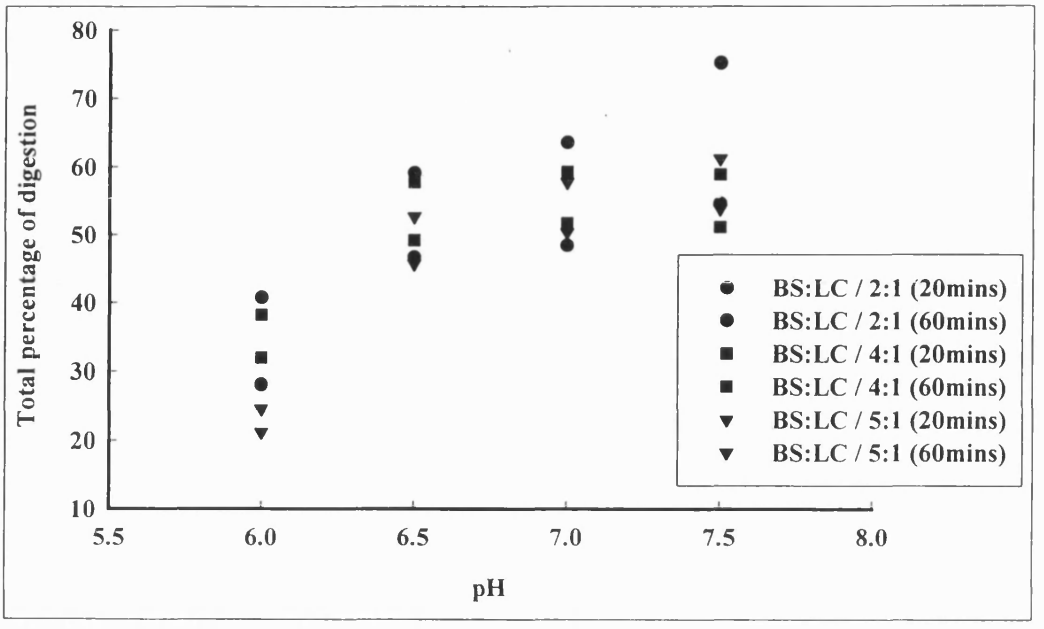


Figure 4.25 The effect of calcium concentration on the digestion profile of corn oil under standard pH-stat conditions

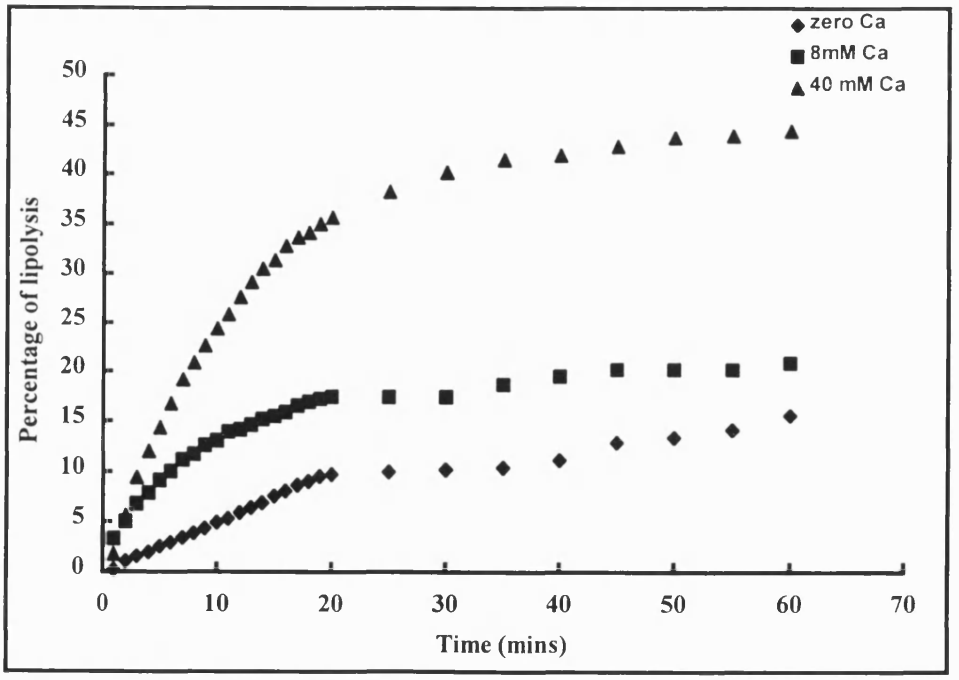
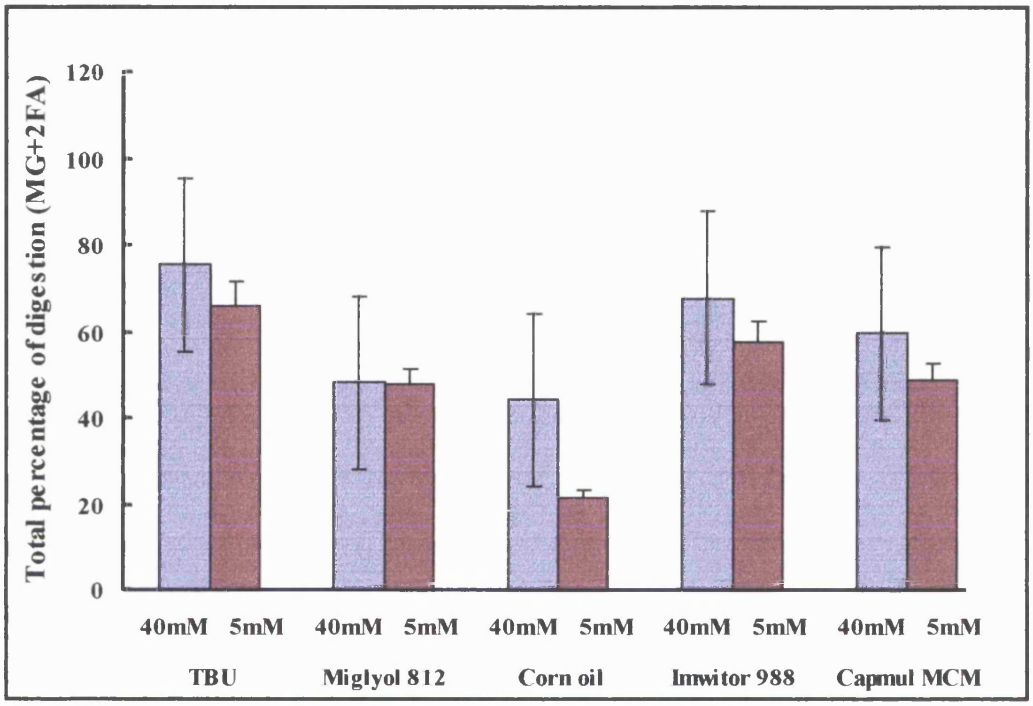


Figure 4.26 Effect of calcium concentration on the rate of lipolysis of mixed mono-, di-, and tri-glycerides, SCT, MCT and LCT using pH-stat method (the error bars represent the standard deviation of three experiments)



Chapter 5

Lipolysis of co-surfactant, surfactants, and lipid formulations in vitro in simulated intestinal fluid.

5.1 Introduction

Fat digestion in the gastro-intestinal tract is likely to have a profound effect on the state of dispersion of lipid formulation and the fate of the drug (MacGregor *et al*, 1997; Pouton, 2000). Lipid formulations for administration of drugs to the gastrointestinal tract generally consist of a drug dissolved in a blend of two or more excipients, which may be natural triglycerides, oils, partial glycerides, surfactants or co-surfactants and water-soluble co-solvents. There are a diverse of delivery systems, with different properties depending on the blend of excipients used. Some are self-emulsifying, some give rise to clear colloidal dispersions on dilution, others do not disperse until they are digested by pancreatic lipase. Whatever the formulation, the fate of the drug *in vivo* is likely to be influenced by whether any of the excipients are digestible, and whether digestion is able to proceed or instead is inhibited by the presence of surfactants.

Pancreatic lipase plays a major role in the lipolysis. The adsorption of pancreatin lipase colipase to the substrate is a fundamental process because the enzyme catalyzes a heterogeneous reaction that involves an interfacial activation step (Hermoso *et al,* 1996). Unlike enzymes acting on soluble substrates, lipolytic enzymes act at the interface between an aqueous medium containing the lipolytic enzyme and the water-immiscible substrates, generally TG (Alvarez *et al*, 1989). Therefore, any compound that can bind to or interact with the interface is able, to some extent, to modify and alter the activity of these enzymes. Because the substrate is generally much smaller than the enzyme, substrate diffusion is rate-limiting. For a water-soluble lipolytic enzyme, the substrate is generally part of an emulsion and/or micelle. These structures may be orders of magnitude larger than the enzyme. Thus, the maximum

rate attainable by a simple Michaelis-Menten mechanism is limited by diffusion of enzyme to the substrate, a process considerably slower than diffusion of monomeric substrate in solution (Brockman *et al*, 1984).

Bile salts, above the range of their critical micellar concentration, inhibit pancreatic lipase activity toward its substrate; this inactivation is reversed by colipase (Borgström, 1975). The inactivation is due to the accumulation of bile salt in the interface, giving it a negative charge and hindering the binding of similar negatively charged lipase to the supersubstrate, thereby also preventing the formation of an enzyme-substrate complex at the catalytic site by increasing the electrostatic repulsion between the substrate and the enzyme (Wickham *et al,* 1998). The addition of colipase to the bile salt-lipase substrate system has, however, a dramatic effect on the binding of lipase to the substrate interface that is parallel to a reversal of the inhibition of the catalytic activity (Borgström *et al*, 1975). The lipase-colipase complex is composed of two more-or-less rigid regions connected by a hinge: one of the regions is formed by the N-terminal domain excluding the flap, and the other is formed by C-terminal domain, colipase, and the flap (Hermoso *et al*, 1996). The pancreatin enzyme adopts an inactive, closed conformation with a surface loop from N-terminal domain (the flap) covering the active site and the colipase has an open conformation resulting from the repositioning of the flap. The motion of the flap makes the active site accessible to the substrate, simultaneously forming a functional oxyanion hole and generating the lipase interfacial binding site. Therefore, once the flap is opened, either by the water-substrate interface or by detergent micelles in the presence of colipase, monomers of nonionic surfactants may behave as inhibitors by binding to the active site. The open conformation has only been observed when lipase was co-crystallized with colipase and either bile salt or phospholipid (Hermoso *et al,* 1996). This, in turn, suggests that binding to the water-lipid interphase may require a certain degree of conformational flexibility. Kinetic data indicate that the binding between colipase and lipase in the presence of substrate is strong and occurs in an approximately stiochiometric relationship (Borgström, 1975). Colipase does

not bind to a pure hydrocarbon interface nor does it affect the binding of lipase to such an interface. It appears that colipase structure provides a more specific binding to a substrate interface and thus directs lipase to its substrate superstructure.

It is well established that the kinetics of lipolysis are simply described by a reversible rate-limiting penetration step of the enzyme into the interface, characterized by a lag time before the steady state reaction is reached (Wieloch *et al,* 1982). Recently, the *in vitro* hydrolysis of phospholipid-emulsified triolein particles, intralipid, by pancreatic lipase in the presence of colipase has shown unusual biphasic kinetics. An initially slow accelerating phase (zero-order kinetics) preceded a sudden phase of high hydrolytic rate (Martigne *et al,* 1987). The lag time is not due to diffusional limitation but rather to slow interfacial penetration of the enzyme (Wickham *et al,* 1998). The duration of the low activity phase was shown to be dependent, among other factors, on the orientation and the concentration of enzyme, and substrate in a surface phase by forces independent of their direct interaction (Brockman *et al*, 1984; Wieloch *et al,* 1982). Because this is a major function related to enzyme-substrate complex formation in homogenous catalysis, direct enzyme-substrate interaction characterized by an interfacial K_m probably used mainly to regulate the specificity of catalysis. The action of a lipolytic enzyme at an interface generates products which can directly activate or inhibit adsorption or catalysis by binding to the enzyme. Nevertheless, the generation of surface-active products can change the interfacial tension, alter lipid packing, change substrate concentration and may even change the amount of surface in the system. The interfacial nature of lipolysis implies that it is the concentration of substrate molecules at the lipid-water interface, which is a direct determinant of rates of lipolysis. Accordingly, it is important, in studying the lipolysis reaction, to understand the physical behaviour of the enzyme-substrate complex. A more relevant consideration with respect to lipolysis is the concentration and the type of the glycerides at the lipid-water interface *i.e.* on the surface of triglycerides-rich fat emulsions.

Diglycerides are more surface-active than triglycerides because of their free hydroxyl groups. Therefore, they can be expected to reside predominantly in surface phases. However, diglycerides do not normally accumulate but are hydrolyzed by most triglyceride lipases to monoglyceride or glycerol and fatty acids. The surface concentration (2–5% of the TG at the interface) of TG decreases with lipolysis of the particle. This indicates qualitatively that the interfacial availability of triglyceride for hydrolysis is a function of the surface composition of the particle, which changes as lipolysis proceeds (Brockman *et al*, 1984).

Therefore, the objectives of the present investigation are:

- To investigate the pancreatin enzyme kinetics acting upon SCT (tributyrin) under the standard pH-stat conditions. The lipolysis kinetics are described through the Michaelis-Menten equation parameters (K_m and V_{max}).
- To study the fate of enzyme activity in the presence of non-ionic surfactant under standard pH–stat conditions.
- To conduct further experiments using SEDDS formulation as substrate under pH–stat conditions, based on the *in-vitro* kinetics studies.

5.2 Materials

In addition to the materials mentioned in chapters, 2, 3, and 4, the following surfactants were used:

Trade name	Scientific name	Dominant fatty acids		Molecular weight	HLB
Labrafil M 1944 CS	Oleoyl Macrogol glycerides. (Apricot kernel oil PEG-6 complex)	C_{16}	4 – 9 %	440	3 - 4
		C_{18}	< 6 %		
		$C_{18:1}$	58 – 80 %		
		$C_{18:2}$	15 – 35 %		
		C_{20}	< 2 %		
		$C_{20:1}$	< 2 %		
Labrafac CC	Medium chain triglycerides (EP)	C_8	65%	327.6	
		C_{10}	35%		
Labrafil M 2125 CS	Linoleoyl Macrogo-glycerides (EP). (Corn oil PEG-6 complex)	C_{16}	4 – 20 %	779.2	3 - 4
		C_{18}	< 6 %		
		$C_{18:1}$	20 – 35 %		
		$C_{18:2}$	50 – 65 %		
		C_{20}	< 1 %		
		$C_{20:1}$	< 1 %		
Labrasol	Saturated Macrogol glycerides EP	C_8	50 – 80 %	469.4	14
		C_{10}	20 – 50 %		
		C_{12}	< 3 %		
		C_{14}	< 1 %		
Gelucire 44/14	Lauroyl Macrogol- 32 glyceride EP	C_8	< 15 %	664.76	
		C_{10}	< 12 %		
		C_{12}	30 - 50 %		
		C_{14}	5 – 25 %		
		C_{16}	4 – 25 %		
		C_{18}	5 – 35 %		

Table 5.1 The polyglycolyzed glycerides surfactant used throughout the investigation. The surfactants were purchased from Gattefossé, s.a, France.

5.3 Determination of the kinetic parameters, K_m and V_{max} for pancreatin acting upon SCT under standard pH- stat conditions

Enzyme properties are determined mainly by means of kinetic studies. The total concentration of enzyme is a constant, which often imposes a great simplicity on the steady–state rate law relating velocity to substrate or to substrate and inhibitor. As a result, the enzymes manifest the well-known Michaelis–Menten behaviour with rectangular – hyperbolic rate law:

$$\upsilon = (V_{max} * S) / (K_m + S) \qquad \text{eq (5.1)}$$

The Michaelis–Menten equation (eq 5.1) relates the rate of substrate consumption or product formation (υ – referred to intial velocity) of an enzyme reaction to the initial substrate concentration (S) present. The kinetics parameters (K_m and V_{max}) describe the shape of a rectangular hyperbola; therefore, K_m and V_{max} are constants. V_{max} is the maximum velocity of the reaction when the substrate concentration is saturating the enzyme. K_m is the Michaelis constant describing the substrate concentration at which the reaction proceeds at half its maximum initial velocity (Morris, 1974; Eisenthal *et al*, 1992).

The derivation of the equation 5.1 depends on three supposition (Eisenthal *et al*, 1974):

- The rate of the reverse reaction is insignificant during the measurement time.
- The measurement is made in the steady state when the concentration of enzyme–substrate complex is unchanging.
- The formation of enzyme–substrate complex will not significantly deplete the concentration of free substrate.

If the previous assumptions are correct, then the intial part of the progress curve representing the enzyme reaction will exhibit linear behaviour. In general, the initial velocity plotted against the substrate concentration and the gradient of the linear portion is taken to the initial velocity as shown in Figure 5.1(Cornish–Bowden,

1996). The concentration of substrate was expressed as the total SCT present in the system. This resulted in a v against s hyperbola with s described as units of millimoles per unit volume. However, the actual concentration of lipid available to the enzyme may not directly relate to the total lipid present. The quantity of lipid partitioned from the bulk into the surface phase or the emulsion droplet size and, hence, interfacial area may vary, limiting availability of substrate for enzyme adsorption. If this is the case, the kinetic parameters may reflect primarily the properties of enzyme adsorption to the interface, not the interfacial interaction of enzyme and substrate. This concern arises because of the non–equilibrium nature of emulsion particles.

Differences in the particle distribution from one preparation to the next can change the value of K_m expressed in molar units (Brockman *et al*, 1984). Under conditions of the pH-stat assay, it is difficult to know how relevant variations in emulsion droplet size would be once lipolysis commenced. The simulated bile solution, along with lipolytic products released, will aid triglyceride emulsification. This, combined with the mechanical stirring action of the pH-stat, could result in emulsion droplets of uniform size irrespective of substrate concentration, thus the effect on the kinetic parameters determined could be minimal.

The plot indicates that at very low concentrations of substrate, the reaction is first order with respect to substrate, and at high concentrations of substrate, υ has a maximum value (V_{max}) and the reaction is zero-order kinetically with respect to substrate (Morris, 1972). As substrate concentration is increased, the enzyme eventually becomes saturated with substrate. In practice, however, it is difficult to draw rectangular hyperbolae accurately, because it is difficult to locate the asymptotes correctly (because one is tempted to place them too close to the curve); it is difficult to perceive the relationship between groups of hyperbolic curves, and to detect deviations from the expected curve if they occur (Cornish–Bowden, 1996). However, the Michaelis-Menten relationship is not directly related to the action of an enzyme upon an insoluble substrate. Therefore, for the kinetics of a lipolytic enzyme

acting at an interface, a model of two separate stages has been proposed, resulting in a two-dimensional form of the Michaelis-Menten equation (Brockman *et al*, 1984). The first stage is an equilibrium to describe the reversible penetration of enzyme into the interface. Once penetration has occurred a second equilibrium exists for the catalytic interaction between a molecule of enzyme and a substrate molecule. These difficulties were recognized by Michaelis–Menten who instead plotted υ against log *a*.

5.3.1 Method

A series of fourteen digestion profiles of pancreatin acting upon SCT (tributyrin) under standard pH–stat conditions were tested. The substrate concentrations were chosen to span at least five times the K_m value. The digestion profiles represented product formed, expressed in terms of fatty acid released in millimoles per unit volume, with respect to time or total percentage digested per volume with respect to time. Each digestion profile was tested to ensure that the initial portion was linear. The initial rate was then calculated from gradient of the initial linear portion that normally occurred during the first six minutes from the start of digestion. Computerized linear regression analysis was applied to calculate the gradient.

5.3.2 Calculation Michaelis - Menten parameters (K_m and V_{max})

5.3.2.1 The Eadie – Hofstee plot

Multiplying both sides of equation 5.1 by υ and rearranging, we obtain the equation straight line plot for the Michaelis – Menten equation

$$\upsilon = V_{max} - (K_m * \upsilon/s) \qquad \text{eq (5.2)}$$

This shows that a plot the initial measurements (υ) against υ / s should be straight line with slope – K_m (-1.9018) and intercepts V_{max} (0.1856) on the υ axis and V_{max} / K_m on the υ/s as illustrated in Figure 5.2 (Cornish–Bowden, 1996). This linear arrangement reveals best fit upon visual inspection between the data and a straight line in order to

check if the equation applies. Figure 5.2 shows a linear relationship (r^2= 0.9904) between substrate concentration (TBU) and initial velocity indicating that the Michaelis–Menten relationship was applicable, despite the heterogeneity of the reaction mixture.

5.3.2.2 The direct linear plot

Eisenthal and Cornish–Bowden (1974) have described a quite different way of plotting the Michaelis – Menten equation, showing the dependence of V_{max} and K_m:

$$V_{max} = \upsilon + (\upsilon / s) * K_m \qquad \text{eq (5.3)}$$

Plotting the substrate concentration used, s, and the initial velocity value, υ, obtained from each digestion profile as an individual pair, creates the direct linear plot. The initial velocity is plotted onto the vertical axis representing the V_{max} and the corresponding substrate concentration s, onto a negative horizontal K_m axis. A straight line is then drawn between the two points and extrapolated into V_{max} and K_m parameters. The resulting lines obtained from each digestion profile should all intersect at the coordinates of the best fit K_m and V_{max} values, if the data fit the Michaelis–Menten equation. In practical each intersection point provides one estimate of K_m and V_{max}, therefore the median of the intersection co-ordinates are taken to the best fit of K_m and V_{max}. The direct linear plot has some advantages over the other because the error in any reading is likely to be positive as negative, the weighting is not relevant, and the method is less sensitive to outliers then least–squares fit method (Eisenthal *et al*, 1992).

Results

A pair of s and υ obtained from each digestion profile of pancreatin acting upon SCT was used to build a direct linear plot as illustrated in Figure. 5.3. A measurement error can be seen from different intersection points. A computer software package was used to calculate the coordinate of each intersection and determine best–fit

median values of K_m and $V_{max.}$ K_m and V_{max} values are summarized in Table 5.2. Clearly, visual inspection of the direct linear plot will not disclose if the data considerably deviate from equation 5.1.

5.3.2.2 Least – squares fit to a hyperbola

A software package was used to calculate the least–square fit of the data points to an initial velocity against the substrate concentration. This method calculates best–fit values of K_m and V_{max} and their standard deviation (Eisenthal *et al,* 1992). The method requires initial estimates of K_m and V_{max} (provided by one of the linear transformation).

The following sequences are summarized when using least-squares fit method for the normal statistical analysis of enzyme kinetics data (Eisenthal *et al*, 1992):

- Random errors in replicate values of the measured velocity, *v*, follow a normal distribution.
- There is no error in the substrate concentration, *s*.
- The correct weightings are known.
- Fluctuations in the *y* values must be independent of any fluctuations in the *x*.

Results

The experimental data from the digestion profile of pancreatin upon SCT (TBU) under standard pH–stat conditions were fitted by using a curve fitting programme. A hyperbolic curve is the result of drawing the initial velocity against the substrate concentration as shown in Figure 5.4. Both K_m and V_{max} values calculated by the least squares method (Table 5.2) are based on the presence of a constant absolute measurement error in the data. Usually, a homogenous error is an inherent assumption of the least squares fit method but it is not necessarily applies for these data. The data exhibit systematic variation of standard deviation, which is dependent on the value of v, known as constant relative error. The addition of the weighting

factors would facilitate the use this heterogeneous error. Therefore, large number of data are required confirming the correct weighting scheme.

5.3.3 Comparison of the analytical method used

The previous method used gave different estimates for K_m and V_{max} when calculated from the same experimental data, which use to within confidence intervals. Therefore, the difference was not significant. Further tests was done for the least-squares fit, with weighting applied to the data which assumed a constant relative error.

Method of analysis	V_{max} (mmol min^{-1})	V_{max} statistics	K_m (mM)	K_m statistics
Direct linear	0.387	0.373 - 0.390 (68 % confidence limits)	11.9	11.1 - 12.9 (68 % confidence limits)
Least squares fit to hyperbola	0.374	0.012 (standard error)	11.1	0.69 (standard error)
Least squares fit to hyperbola with constant relative error	0.391		12.1	

Table 5.2 The value of V_{max} and K_m for pancreatin acting upon SCT under standard pH – stat conditions) calculated using different analytical methods.

Using a constant relative error gave for V_{max} and K_m close to those given by the more statistically robust direct linear method. As a result, the data exhibit a heterogeneous error. The increase in the measured error was proportional to the increase of the velocity values (Table 5.2). The most reliable was the direct linear plot because of the absence of the weighting scheme. On the other hand, many data points were necessary in case of the least-square fit method to confirm the correct weighting scheme. The precise estimates of V_{max} and K_m would have standard deviations of less

than 10% of the mean V_{max} and K_m values, as mentioned by Cleland (reviewed by Eisenthal *et al*, 1992). As in the case of the direct linear plot, the standard deviation in terms of the percent of the mean is 7.7% for K_m and 3.11% for V_{max}, and the values from the least squares fit method are 6.51% for K_m and 1.98% for V_{max}. The good fit of the experimental data to equation 5.1 increased the similarity of K_m and V_{max} values derived from the two analytical methods. Therefore, the choice of analytical method would probably not alter conclusions drawn from the use of K_m and V_{max} values in subsequent experiments.

5.4 Lipolysis of lipid formulations

5.4.1 Kinetics

Preliminary studies used pure triglycerides and lipid excipients as substrates. In the subsequent investigations the substrates were lipid formulations; combinations of mixed glycerides (mono-, di-, and tri–glycerides), MCT and /or surfactant with high or low HLB value. The digestion profiles were followed by continuously titrating the liberated fatty acids over time under standard pH–stat conditions as explained in chapter 4.

Throughout the research, an assumption was used to calculate the number of fatty acids released in each lipid formulation. Table 5.3 explains the main criteria used to estimate the FA expected to be released from mixtures of mono-, di- and tri-glycerides (Imwitor 988®) and MCT (Miglyol 812®). In the presence of the surfactant the assumption made was different. Release depended mainly on the HLB of the surfactant. To allow assessment of the extent of inhibition the maximum expected liberation of FA was estimated. Table 5.4 represents the expected FA release from Miglyol 812® blended with 100% TG and Tagat TO® as lipophilic surfactant (HLB= 11.3). For the purpose of estimation Tagat TO® was considered as 45% TG.

mass of MCT Miglyol 812®	mass of mixed glycerides Imwitor 988®	moles of TG*	moles of DG*	moles of MG*	moles FA expected **	max moles FA ***
1.0	0.0	0.00200	0.00000	0.00000	0.00400	0.00600
0.9	0.1	0.00180	0.00013	0.00027	0.00373	0.00593
0.8	0.2	0.00160	0.00026	0.00054	0.00346	0.00586
0.7	0.3	0.00140	0.00039	0.00081	0.00319	0.00579
0.6	0.4	0.00120	0.00052	0.00108	0.00292	0.00572
0.5	0.5	0.00100	0.00064	0.00135	0.00264	0.00563
0.4	0.6	0.00080	0.00077	0.00162	0.00237	0.00556
0.3	0.7	0.00060	0.00090	0.00189	0.00210	0.00549
0.2	0.8	0.00040	0.00103	0.00216	0.00183	0.00542
0.1	0.9	0.00020	0.00116	0.00243	0.00156	0.00535
0.0	1.0	0.00000	0.00129	0.00270	0.00129	0.00528

* RMM TG= 500 **expected *** max.

RMM DG = 358 TG 2 FA 3 FA

RMM MG = 200 DG 1 FA 2 FA

MG 0 FA 1 FA

Table 5.3 The assumption made to calculate the FA released from the lipolysis of lipid formulations.

mass of MCT Miglyol 812®	mass of lipophilic surfactant Tagat TO®	moles of TG from M 812®	moles of TG* from TTO®	moles FA expected **	max moles FA ***
1.0	0.0	0.00200	0.00000	0.00400	0.00400
0.9	0.1	0.00180	0.00009	0.00360	0.00378
0.8	0.2	0.00160	0.00018	0.00320	0.00356
0.7	0.3	0.00140	0.00027	0.00280	0.00334
0.6	0.4	0.00120	0.00036	0.00240	0.00312
0.5	0.5	0.00100	0.00045	0.00200	0.00290
0.4	0.6	0.00080	0.00054	0.00160	0.00268
0.3	0.7	0.00060	0.00063	0.00120	0.00246
0.2	0.8	0.00040	0.00072	0.00080	0.00224
0.1	0.9	0.00020	0.00081	0.00040	0.00202
0.0	1.0	0.00000	0.00090	0.00000	0.00180

* TTO considered 45 % as TG

** TG = 2 FA (produced from M 812® only)

*** TG = 2 FA(M 812®)+ 2 FA (TTO®)

Table 5.4 The assumption made to calculate the FA released from the lipolysis of type II formulations (Miglyol 812® and Tagat TO®).

mass of mixture Miglyol 812 and Imwitor 988® (1:1)	mass of lipophilic surfactant Labrafil M® 1944 Cs	moles of TG from M 812®	moles of DG from I 988®	moles of MG from I 988®	moles of TG* from L M®1944	moles FA expected **	max moles FA ***
1	0	0.00100	0.000645	0.00135	0.00000	0.00265	0.00564
1	0.2	0.00167	0.000536	0.00112	0.00043	0.00388	0.00806
1	0.4	0.00143	0.00046	0.00096	0.00073	0.00332	0.00763
1	0.6	0.00125	0.00040	0.00008	0.00096	0.00290	0.00655
1	0.8	0.00111	0.000357	0.000075	0.00114	0.00258	0.00640
1	1	0.00100	0.000246	0.000067	0.00128	0.00225	0.00612

* TG = 2 FA from Labrasol M® 1944 Cs

	**expected	*** max.
TG	2 FA	5 FA
DG	1 FA	2 FA
MG	0 FA	1 FA

Table 5.5 The assumption made to calculate the FA released from the lipolysis of type IIIA in the presence of Labrafil M® 1944 CS (HLB 4–6) as hydrophilic surfactant.

mass of mixture Miglyol 812® and Imwitor 988® (1:1)	mass of lipophilic surfactant Cremophor RH 40®	moles of TG from M 812®	moles of DG from I 988®	moles FA expected
1	0	0.00100	0.000645	0.00265
1	0.2	0.00167	0.000536	0.00388
1	0.4	0.00143	0.00046	0.00332
1	0.6	0.00125	0.00040	0.00290
1	0.8	0.00111	0.000357	0.00258
1	1	0.00100	0.000246	0.00225

Table 5.6 The assumption is made to calculate the FA released from the lipolysis of type IIIA in the presence of Cremophor RH 40® (14–16) as hydrophilic surfactant.

Tables 5.5 and 5.6 represent the expected difference in the total fatty acids that could potentially be released from different groups of surfactant. For example, Table 5.5 showed that the expected lipolysis in the presence of polyglycolized glycerides like Labrafil M® 1944 Cs was estimated for glycerides and MCT. Other experiments required estimation of available fatty acid esters in blends of Cremophor RH 40®, an example of hydrogenated castor oil ethoxylates as shown in Table 5.6.

In general, the fatty acids liberated were calculated on the basis of 2-FA from TG, 1-FA from DG and 1 FA from MG. Although there is another possibility that MG produced from TG give another 1-FA.

5.4.2 The fate of the lipid formulations upon lipolysis in the presence non - ionic surfactants

Hydrolysis rates of SEDDS displayed saturation kinetics with respect to total oil (substrate) concentration. The results of the lipolysis of SEDDS formulations under standard pH-stat conditions are displayed in Figures 5.4-5.13. The saturation data did not obey the typical Michaelis–Menten equation. The increase in the rate with the lipolysis was faster than expected from a square hyperbola. Figure 5.5 represents the profile of digestion over 60 min of type II formulations {MCT (Miglyol 812®) and lipophilic surfactant (HLB<12) (Tagat TO®)} under standard pH – stat conditions. Examination of the resulting profiles from increasing concentration of TTO® suggested some suppression of pancreatin activity. This was evident from the total TG digested upon completion of the assay. At 40% of MCT in the presence of TTO® (60%) the percentage digested was <30% as illustrated in Figure 5.6. Whereas at 40% of MCT, alone, the percentage digested was <35%. This effect could be explained by the phase separation of the oil and the surfactant (alkylphenol) on mixing with water to form two populations of droplets (one rich in MCT oil and the other in alkylphenol) only one of which enables lipolysis to proceed.

Figure 5.7 show the fate of digestion over time of a fixed ratio of MCT (Miglyol 812®) and mixed mono-, di-, and tri-glycerides (Imwitor 988®)(1:1) when CRH 40® (HLB 14–16) was added gradually under the standard pH-stat conditions. The lag phase was more obvious in the presence of CRH 40® than in the presence of TTO® or Labrafil M1941® as shown in Figure 5.8. The surfactant would be expected to be anchored at the oil-water interface. Therefore, it is likely that the lipolysis was inhibited once the oxyethylene mantle generated by the surfactant exceeded a critical thickness, which prevented bonding of colipase-lipase complex to the surface of the oil droplet. Figure 5.9 illustrated that the total percentage of digestion was significantly affected by the addition of CRH 40®. At 20% of CRH 40® did not reduce the lipolysis of the mixed glycerides and MCT. At 50% of CRH 40® the effect upon the pancreatin activity was more clear as shown in Figures 5.7 and 5.9.

Figure 5.9 shows the difference in the total of digestion fixed ratio of MCT (Miglyol 812®) and mixed glycerides mono-, di-, tri-glycerides (Imwitor 988®)(1:1) upon addition of surfactant with different HLB. Polyglycolized glycerides like Labrasol M®1944 (HLB 3–4) produced a greater than expected increase in the total percentage of digestion over a longer period of time as shown in Figure 5.10. The long chain polyglycolized glycerides (6-PEG $C_{18:1}$ and $C_{18:2}$) depressed pancreatin activity at steady state with a decrease in total MCT digested upon completion of the assay compared to the control (Solomon, 1998). Solomon (1998) mentioned that the $C_{18:2}$ 8-PEG had similar behaviour with the short lag phase, probably due to higher hydrophilic character compared to the 6-PEG variant. The inhibition of pancreatin activity by the $C_{18:1}$ and $C_{18:2}$ polyglycolized glycerides had a different behaviour of inhibition other than more hydrophilic surfactants. This may be due to the fact that the long-chain polyglycolized glycerides are composed of natural oil in a complex with PEG. On the other hand, the medium-chain polyglycolized glycerides ($C_{8:1}$) showed little inhibition relative to their HLB numbers of 10 and 14, values for which other surfactant classes resulted in extensive enzyme inhibition. This may be a result of the composite nature of these products.

Mixed mono-, di- and tri–glycerides (Imwitor 988®) and MCT (Miglyol 812®) were studied as a control experiment as presented in Figure. 5.11. During product activation lipolysis, the enzyme initially partitions between the bulk water and lipid interface. Hydrolysis starts and products temporarily remain at the interface, forming clusters or microheterogeneities. At the boundaries of these clusters, the binding of the enzyme is enhanced with resultant increase in hydrolytic rate (Wieloch *et al*, 1982).

The fate of lipid formulations under standard pH–stat conditions is characterized by three distinct phases. In the activation phase or lag phase, the rate of reaction increases continuously until a maximum rate is reached. At this point it has been suggested that lipase and colipase bind to LC-BS micelles in solution. In the zero–order or initial–rate phase, the rate of FA production is linear with time. Linear kinetics is explained by an increased binding of lipase and colipase to the substrate surface, induced by the free fatty acids formed. The final phase is characterized by a continuous decrease in rate until the reaction stops, when all possible FA has been released or when the rate of FA formation becomes negligible. The presence of an activator phase and a lag phase depended on the nature of TG present in lipid formulation. In general, LCT and hydrophilic surfactant with high HLB value had a more pronounced and longer lag phase than MGT, SCT, and hydrophilic surfactant with low HLB value. Figure 5.12 illustrates the fate of different lipid formulations under the same standard pH – stat conditions. The three phases are characterized in each formulation.

The lag phase was more pronounced in type IIIA than in type II and S-F formulation (Figure 5.12). The lag phase is due to the presence of CRH 40® (<35%) in type III A that inhibits the lipolysis. A possible explanation for their higher inhibitory effect may be due to strong attractive forces between the substantial castor oil component and MCT. Resultant adsorption of the hydrophobic castor oil moiety of the surfactant to the lipid interface could restrict binding of enzyme to the substrate, if surfactant concentration was sufficient to cover the entire interface. The castor oil has three

sites where ethoxylation is most likely to occur (on the hydroxyl groups), with a possibility of ethoxylation also at the site of the three carbonyl groups. In the case of alcohol ethoxylates all the ethoxy groups form a single chain upon one site. Therefore, if the inhibitory effect is due to a barrier formed by ethoxy groups at the lipid interface, the castor oil ethoxylates would require a higher degree of ethoxylation to build up the length of the ethoxy chains, and thus reach the same inhibitory effect as an alcohol ethoxylate with a lower ethoxylation number.

Figure 5.12 shows that surfactant-free formulations typically follow the hyperbolic curve with no lag phase. The presence and type of co-solvent has no effect on the rate of lipolysis as illustrated in Figure 5.13.

Some experiments were run using the non–ionic surfactant as substrate under the standard pH–stat conditions. Table 5.7 summarized the total amount of FA released from each surfactant varied in accordance with HLB value and their chemical structure. Therefore, the total digestion over time (60 min) was varied. Polyglycolized glycerides (like Labrafils and Labrafac) with low HLB underwent lipolysis as if mixed glycerides. Hydrogenated castor oil ethoxylated (like CRH 40® and TTO®) with high HLB did not undergo lipolysis. Labrasol® had a different behaviour from the other examples that undergo lipolysis with high HLB value. This may be explained by its synthesis by trans-esterfication, which results in the presence of free triglyceride (and free PEG).

Surfactant	HLB	% of digestion 60 min
Gelucire 44/ 14®		4.653
Labrafil M® 1944	3 – 4	26.550
Labrafil M® 2522	3 - 4	36.567
Labrafac CC®	10	5.574
Labrasol®	14	91.063
Tagat TO®	11.3	zero
Cremophor RH 40	14 - 16	zero

Table 5.7 The percentage of digestion of non-ionic surfactant under standard pH–stat conditions.

5.4.3 Discussion

When pancreatin acted on lipid formulations under conditions of the standard pH-stat assay, no lag phase was observed. The supramicellar bile salt concentrations in the simulated bile solution would be expected to inactivate lipase by causing enzyme displacement from the lipid interface (Borgström, 1976). However, colipase present in pancreatin may have been able to overcome any desorbing activity of bile salts resulting in immediate lipase activity, possibly by acting as an anchor for lipase to bind to the substrate (Borgström, 1977a).

The process of colipase binding to the lipid substrate has been suggested to occur *via* hydrogen bonding with ester linkages in the lipid substrate (Borgström *et al*, 1984). The hydrogen bonds have been reported to be easily disrupted by non-ionic surfactants and octylphenol ethoxylate has been demonstrated to displace colipase from the lipid substrate interface. Tensioactive agents, such as non-ionic surfactants, have also been stated to mediate inhibition of lipase by affecting the interfacial quality of the substrate (Piéroni *et al*, 1990).

In the work presented here, pancreatin has demonstrated the ability to overcome the initial lag phase caused by the presence of polyoxyethylene glycerol fatty acid ester

(TTO®), although the times taken to reach steady state did vary. This initial inhibition of enzyme activity upon commencement of the assay may be a direct result of surfactant altering the quality of the substrate interface. Colipase, whilst able to overcome any desorption of lipase due to bile salts in the reaction milieu, may be unable to overcome immediately effects of surfactant molecules.

Surfactant molecules could initially be assumed to accumulate at the lipid interface blocking adsorption of colipase, possibly by steric hindrance or interference with hydrogen bonding between colipase and triglyceride substrate. Eventually, binding of some colipase would allow limited lipolysis to proceed. The resulting accumulation of lipolytic products at the interface would thus promote further colipase binding and increase enzymatic activity to steady state.

Studies by other workers suggested that colipase rather than lipase is responsible for overcoming inhibition from non-ionic surfactants. Colipase has been shown to activate lipase in the presence of substrate interfaces to which lipase cannot bind alone (Verger *et al*, 1977). The addition of colipase in increasing amounts to a detergent inhibited system has also been demonstrated to eventually abolish the lag phase (Borgström, 1977c).

If the ability of colipase to restore lipase activity is related to length of the lag phase, the duration of lag phase must reflect the effect of surfactants on the binding of colipase. Solomon (1998) suggested after an experiment using MCT / nonylphenol ethoxylated surfactant mixtures, that digestion profiles from one batch of pancreatin had a shorter lag phase compared to a second batch. This implies that batch I pancreatin contained colipase with higher activity than batch II.

The literature goes some way to supporting this assumption, as the structure of colipase has been found to be related to the duration of the lag phase. Colipase isolated from porcine pancreatic glands can vary in the number of amino-acid residues present in the peptide chain. Proteolysis at the N-terminus will result in conversion of procolipase (colipase-101) to the more active colipase-96 form

(Borgström *et al*, 1984). When acting upon phosphatidylcholine-stabilized triglyceride emulsion, colipase-96 can overcome lag times at 100 times lower concentrations than procolipase (Borgström *et al*, 1979). In addition cleavage of colipase-96 at the C-terminal end to colipase-85 reduced lag time further by a factor of six (Larsson *et al*, 1981).

Hermoso and his co-workers (1996) reported extensive studies designed to investigate the structural behaviour of porcine pancreatin enzyme in the presence of the non-ionic surfactant tetraethylene glycol monooctyl ether (TGME) and diisopropyl 4-nitrophenyl phosphate (E600). The former surfactants were compared with NaTDC, and ionic surfactant. They clearly demonstrated that the presence of micelles of either ionic or non-ionic surfactant plus colipase induces the opening of the flap exposing the active site of the lipase. The presence of the TGME molecule in the active site provided a structural basis for the inhibitory effect of the surfactant. Therefore, once the flap is opened, either by water-substrate interface, or by detergent micelles in the presence of colipase, monomers of non-ionic surfactant may behave as inhibitors by binding to the active site. In contrast to the inhibition induced by bile salts, non-ionic surfactant inhibition cannot be reversed by colipase (Hermoso *et al*, 1996).

Solomon (1998) reported an extensive study of pancreatin activity on MCT in presence different types of non–ionic surfactants. She mentioned that alteration in the activity of pancreatin by nonylphenol ethoxylated surfactants (NPEs) appeared to be related to the degree of ethoxylation of the surfactant and, hence, surfactant hydrophile-lipophile balance. This may perhaps be explained by consideration of the behaviour of NPEs at a lipid-water interface. The orientation of nonylphenol ethoxylated surfactant molecules at an oil-water interface has been suggested to vary depending upon length of the ethoxy chain, as represented in Figure 5.1.

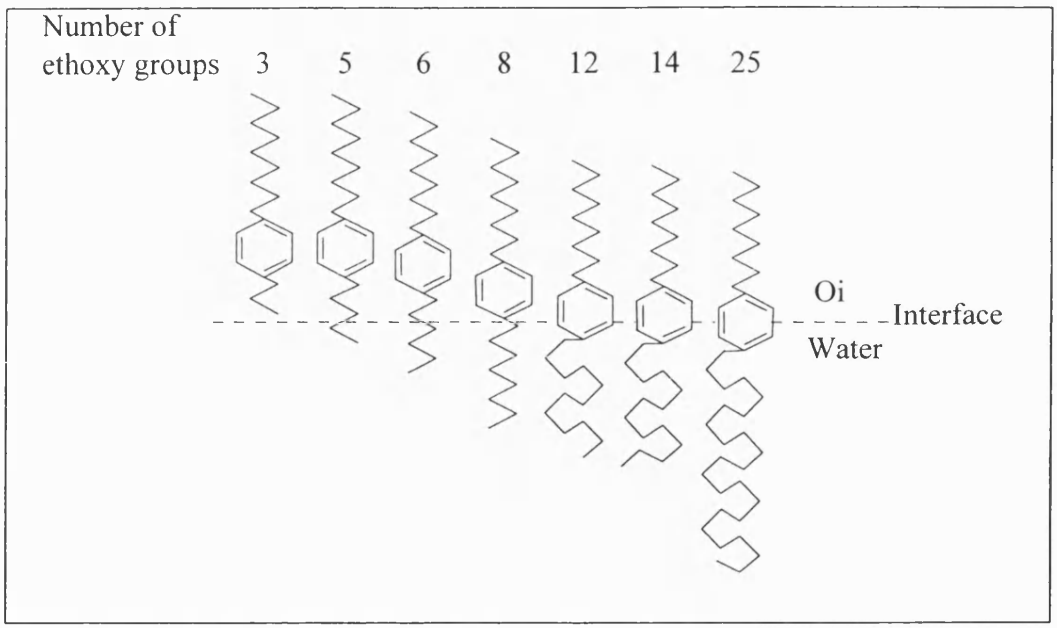


Figure 5.1 The state of orientation of NPEs with different numbers of ethoxy groups at an oil-water interface (modified from Marszall, 1987).

For NPEs with high lipophilic character, the surfactant molecule is represented as completely partitioned into the oil phase as illustrated in Figure. 5.1. As the number of ethoxy groups in the chain is increased from 5 to 8, the hydrophilic ethoxy chain gradually penetrates into the aqueous phase until only the nonylphenol moiety remains in the oil. Additional ethoxylation after this point results in the ethoxy chain adopting a meander form in the water with the hydrocarbon group also penetrating further into the aqueous phase.

In the standard pH-stat assay, triglyceride droplets, probably with associated lecithin, are dispersed in an aqueous reaction mixture as oil-in-water emulsion. The nonylphenol ethoxylated surfactant molecules are assumed to be located mainly at the interface between the triglyceride droplet and the aqueous environment. If the surfactant molecules adopt a similar conformation to that illustrated in Figure 5.1 according to the length of their ethoxy chain, a mechanism to explain the change in their inhibitory effects with HLB can be proposed (Solomon, 1998).

Nonylphenol and NPEs with ethoxy chain lengths below 5 have dominant lipophilic character. From Figure 5.1, the highly lipophilic nature of these surfactants would result in the complete molecule being partitioned into the triglyceride droplet. The lipase and colipase would still be able to bind and start digestion immediately as demonstrated by the lack of a lag phase at the beginning of the digestion profiles produced in the presence of these surfactants. Penetration of the surfactant completely into the oil may cause a substrate dilution effect by acting as inert spacers, resulting in less triglyceride available at the interface for lipase to act upon. This is analogous to the concept of substrate dilution used in lipolytic enzyme kinetic studies where a lipid or detergent molecule known not to be a substrate for the enzyme is introduced into the system (Piéroni *et al*, 1990).

The digestion profiles support the substrate dilution theory by exhibiting decreased enzyme activity at steady state and a reduction in the extent of triglyceride digestion achieved within the assay period as in the presence of Tagat TO®.

For NPEs and ethoxylates of hydrogenated castor oil with chains containing from 5 to 15 ethoxy groups, the potency of inhibition increases with the degree of ethoxylation of the surfactant. Chains composed of 6 to 8 ethoxy groups will start to form an oxyethylene mantle around the lipid droplet, restricting access of colipase to the interface and thus delaying enzyme adsorption (Solomon, 1998). As the ethoxy chain is increased to 9 groups and above, the mantle will become harder to penetrate as the ethoxy chain adopts a meander form. This accounts well for the increase in duration of the lag phase with the degree of ethoxylation of the surfactant as in the presence of CRH 40®, reflecting restricted colipase binding to substrate. It also illustrates the dependence of lipase activity on the ability of colipase to overcome the presence of surfactant, thus revealing the advantage of colipase with higher activity.

The potency of inhibition of NPEs with ethoxy chain lengths of above 15 starts to diminish as hydrophilic character of the surfactant is increased by lengthening of the ethoxy chain. Figure 5.1 illustrates how partitioning of the nonylphenol moiety

further into the aqueous reaction system is likely due to the dominant hydrophilic character of the molecule. These highly hydrophilic surfactants may show reduced inhibition due to preferential partitioning of the surfactant from the lipid interface into the aqueous reaction medium. This would result in enhanced colipase binding to the lipid interface and a subsequent increase in the rate and extent of MCT lipolysis.

The polyglycolized glyceride (C_8-C_{10}) Labrosol is present with free glycerides and probably free polyethylene glycol as well. As the surfactant is added to the assay by weight, the mixture of constituents will have reduced the concentration of inhibitory substance present within the reaction system. Free polyethylene glycol would be expected to partition into the aqueous phase. More importantly the free medium chain glycerides could be assumed to be available at the lipid interface for hydrolysis by pancreatin and to enhance binding of colipase according to the colipase partitioning theory. This may explain the short lag phase seen and a final extent of triglyceride digestion equivalent to that shown by the MCT control.

In general, the chemical structure of the surfactant, in addition to the HLB value, was related to the potency of inhibition of a surfactant. The pattern of inhibition shown across a digestion profile had a particular character depending upon whether the lipophilic or hydrophilic moiety of the surfactant was dominant. This, in turn, suggested inhibition to be mediated by different mechanisms.

The high potency of inhibition demonstrated by the castor oil ethoxylates is suggested to relate to the extensive hydrophobic castor oil component. In all cases digestion profiles from the castor oil ethoxylates (Cremophor RH 40®) displayed a lag phase, indicating an initial delay in enzyme activity. Suppression of enzyme activity across the whole profile has been proposed to be a result of incorporation of surfactant molecules into the interface, where they effectively dilute the concentration of substrate available at the interface. The large size of the castor oil component and the close proximity of the ethoxylation sites to the fatty acid chains may prevent integration of these surfactants into the triglyceride interface. Instead, a surfactant

layer may be formed upon the MCT surface thus preventing initial enzyme / substrate binding resulting in a lag phase.

5.5 Conclusion

It is clear that, under conditions of the standard pH-stat assay, a relationship exists between the hydrophile-lipophile balance of a surfactant and the ability of the surfactant to inhibit pancreatin activity towards MCT and mixed glycerides. At low HLB values surfactants have been proposed to inhibit pancreatin *via* lipophilic effects whereas when HLB is increased by ethoxylation hydrophilic inhibitory mechanisms may come into play. However, the results suggest that HLB not be the only factor involved, as the structure of the hydrophobe also appears to have an influential effect on the potency of inhibition shown by a surfactant. In general, for surfactants with the same extent of ethoxylation, the inhibitory effect appears to increase with the size of the hydrophobic moiety.

It could be argued that the overall size of the surfactant molecule when present at the lipid-water interface may alone be sufficient to inhibit lipase purely by preventing access of colipase to the substrate. The altered nature of the digestion profiles according to HLB value of the surfactant, however, tends to suggest the situation is more complex. For surfactants with dominant hydrophilic character, the effect upon digestion of MCT and mixed glycerides is to delay initial enzyme activity as indicated by a lag phase on the digestion profile. The lag phase is subsequently overcome after a variable period of time, which tends to increase with the hydrophilic character of the surfactant.

Conversely, the lipophilic surfactants, except the castor oil ethoxylates, decreased enzyme activity at steady state without evidence of a lag phase. Inhibition in this case was proposed not to be mediated by surface activity but to relate to a substrate dilution effect with surfactant molecules acting as inert spacers, reducing triglyceride concentration available at the interface for hydrolysis.

Lipophilic surfactants also tended to cause a reduction in the percentage of triglyceride digested upon completion of the assay compared to that shown by lipid formulation control. This may be result of low HLB surfactants forming two phases of droplets, one of which is rich in surfactant. Any formulation formed of mixed glycerides and MCT incorporated in this phase could be expected to be held in a form unavailable to pancreatin, thus explaining the reduced level of triglyceride digestion observed at the end of the profile.

Throughout this study the mechanism of inhibition has been related to surfactants altering the availability of the substrate to the enzyme. A further point to remember is that mono-, di-, and tri- glycerides / MCT / surfactant emulsion systems could be expected to have different interfacial areas depending upon the HLB value of the surfactant, which could also influence pancreatin activity to some extent. The possibility of surfactant monomers in solution having a direct influence on the catalytic properties of lipase also cannot be ignored; some detergent molecules have been observed in crystallographic studies to locate at the entrance of the active site of lipase, bound to the hydrophobic part of the lid (Egloff *et al*, 1995).

Figure 5.2 Representative model of the Michaelis-Menten equation (Eadie-Hofstee plot v versus v/s)

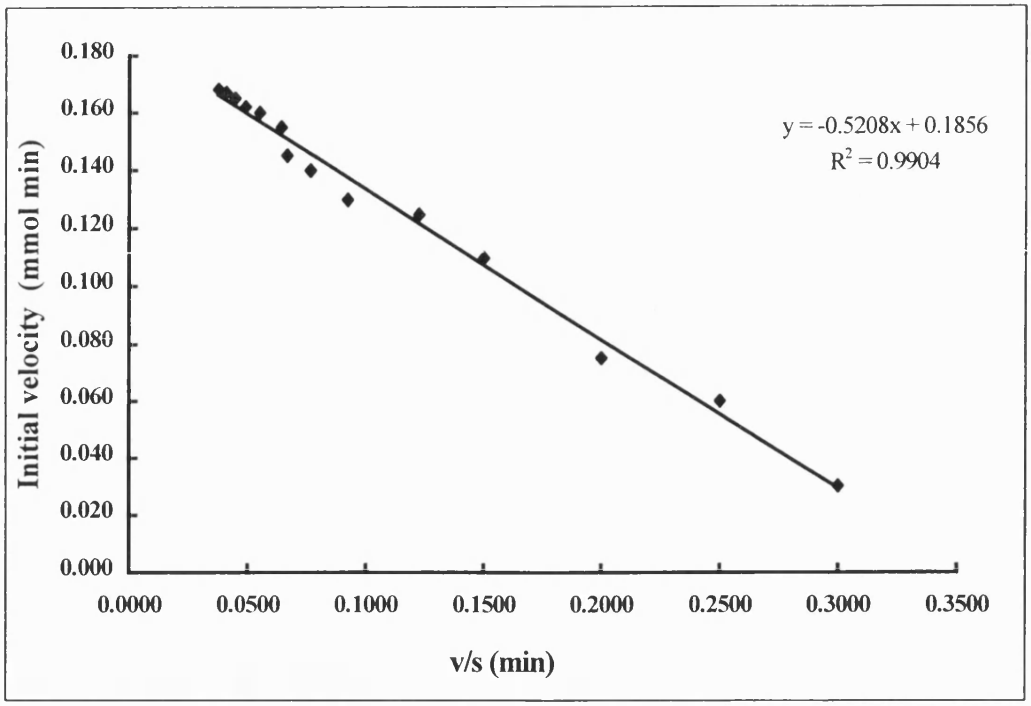


Figure 5.3 Direct linear plot for the digestion of SCT by pancreatin using pH-stat method (V_{max} and K_m are determined from the best fit median values of the intercepts)

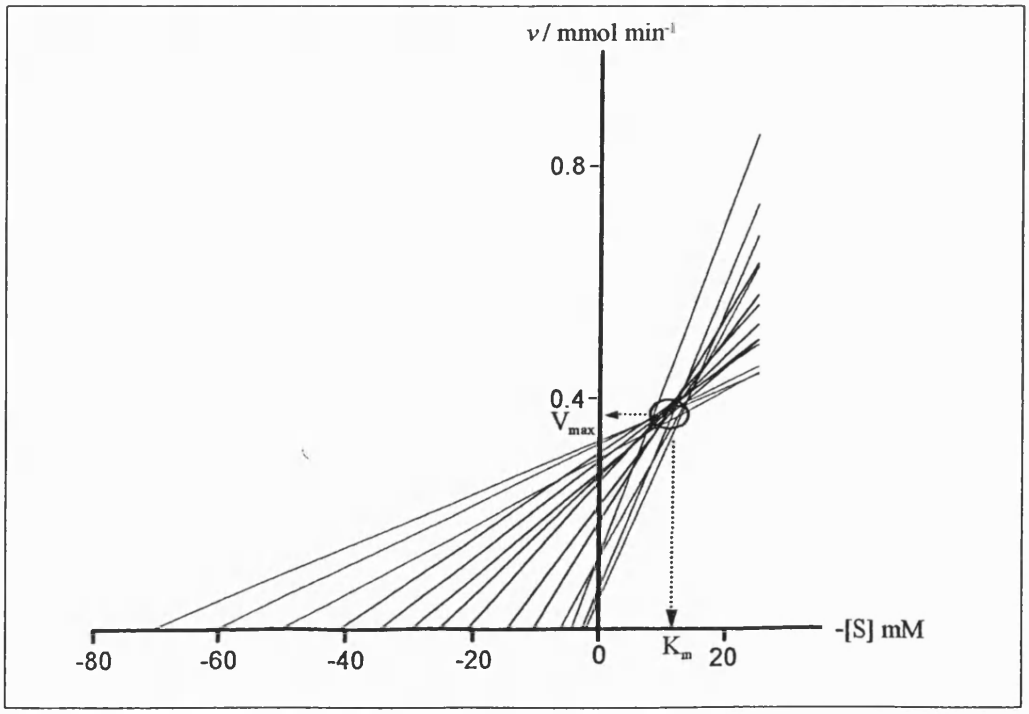


Figure 5.4 Representative hyperbolic curve of the hydrolysis of SCT by pancreatin

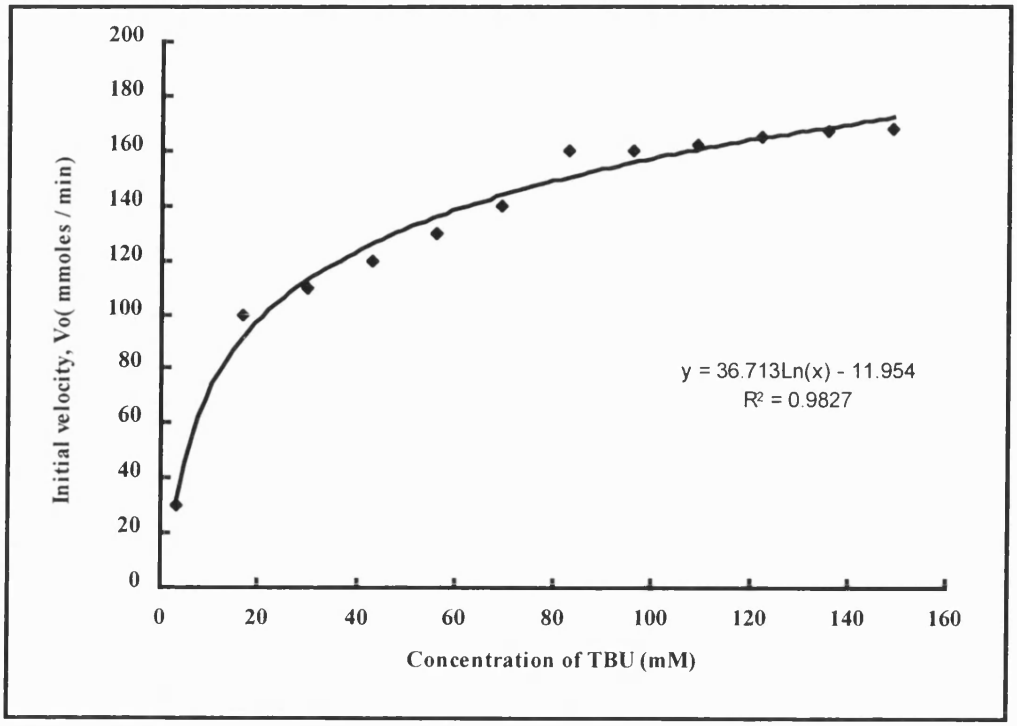


Figure 5.5 Profile lipolysis of type II formulation (Miglyol 812 : Tagat TO) under standard pH-stat conditions

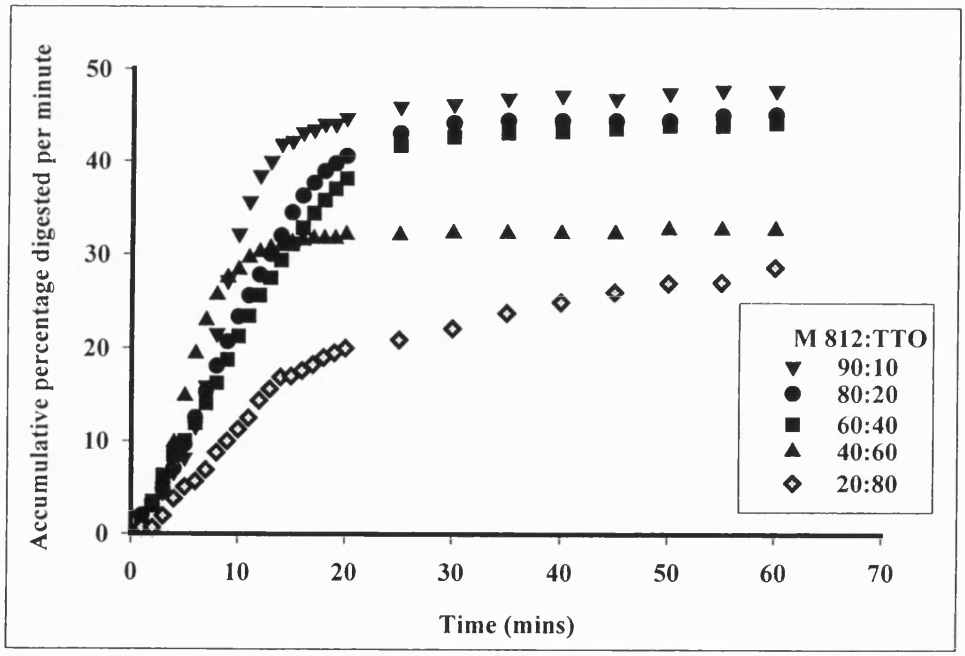


Figure 5.6 Total digestion of type II formulations (Miglyol 812:Tagat To) under standard pH-stat conditions (the error bars represent the standard deviation of three experiments)

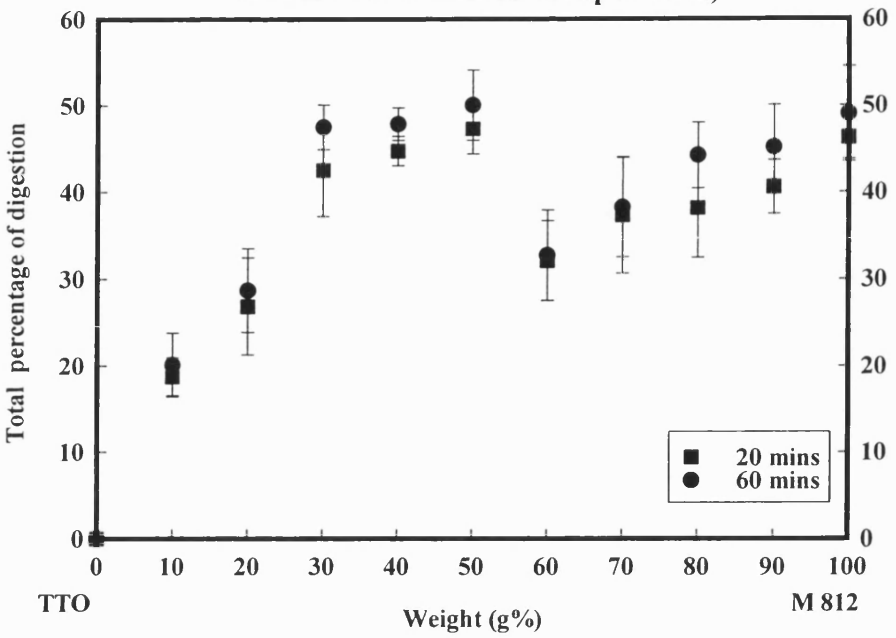


Figure 5.7 The effect of adding Cremophor RH 40 (HLB = 14- 16) on the profile of lipolysis of mixtues of medium chain triglycerides and mono-, di-, tri-glycerides (Miglyol 812 : Imwitor 988) (1:1) under standard pH-stat conditions

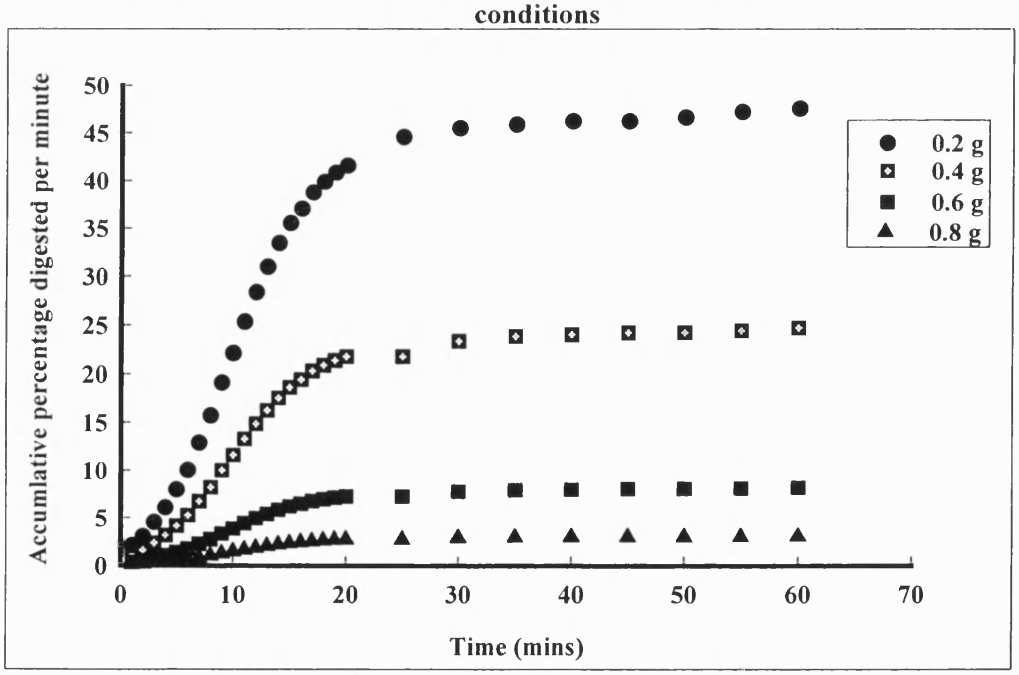


Figure 5.8 Representative examples of the fate of mixtures of medium chain triglycerides and mono, di, and tri-glycerides (Miglyol 812 : Imwitor 988) (1:1)in the presence of surfactant (1g) under standard pH-stat conditions

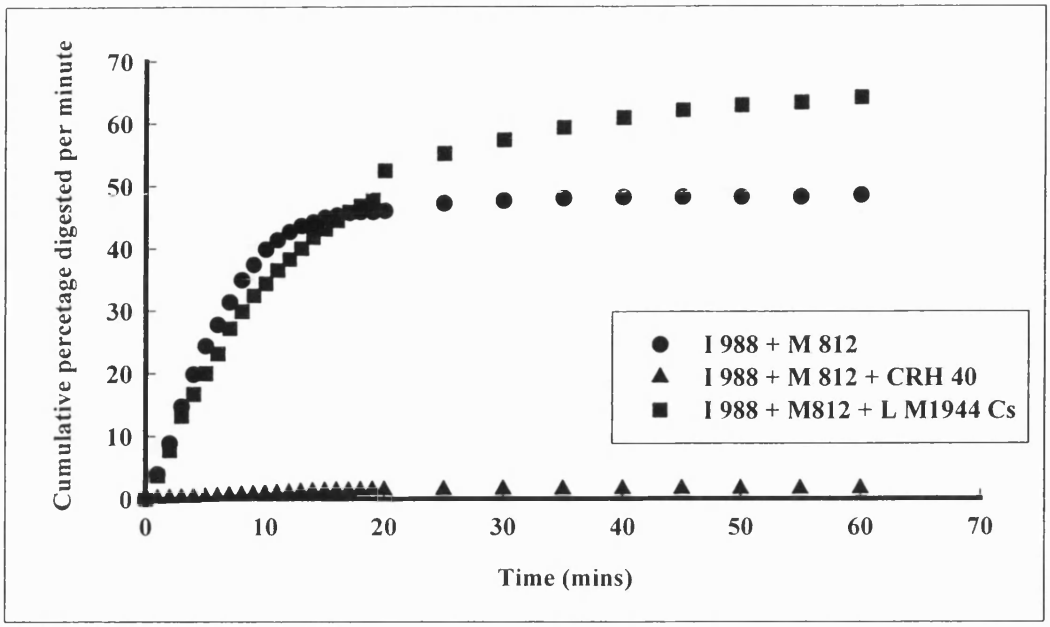


Figure 5.9 The effect of surfactant with different HLB value on the total percentage digested of mixture of medium chain triglyceride and mono, di, and tri-glycerides (Miglyol 812:Imwitor 988) (1:1) under standard pH stat conditions (the error bars represent the standard devaition of three experiments)

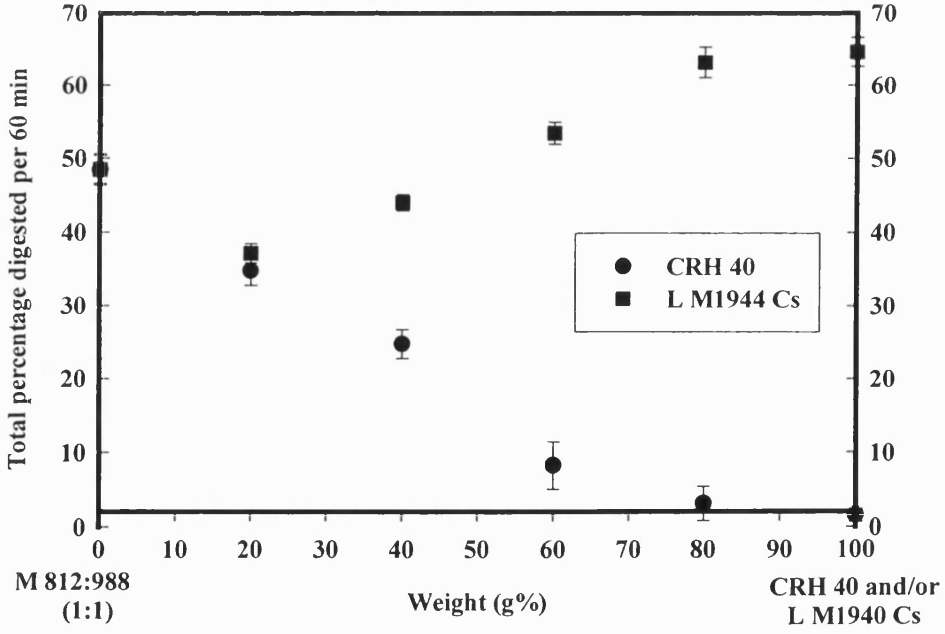


Figure 5.10 The effect of adding Labrsol M1940 (HLB = 4-6) on the profile of lipolysis of mixtues of medium chain triglycerides and mono, di, tri-glycerides (Miglyol 812 : Imwitor 988) (1:1) under standard pH-stat conditions

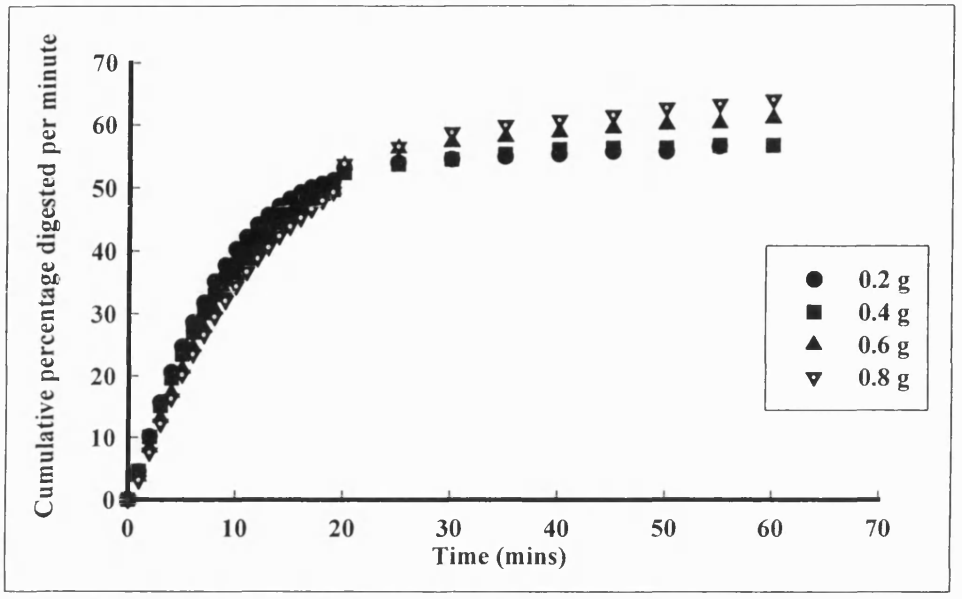


Figure 5.11 Total digestion of mixtures of medium chain triglycerides and mono-, di-, and tri-glycerides formulations (Imwitor 988:Miglyol 812) under standard pH-stat conditions (the error bars represent the standard deviation of three experiments)

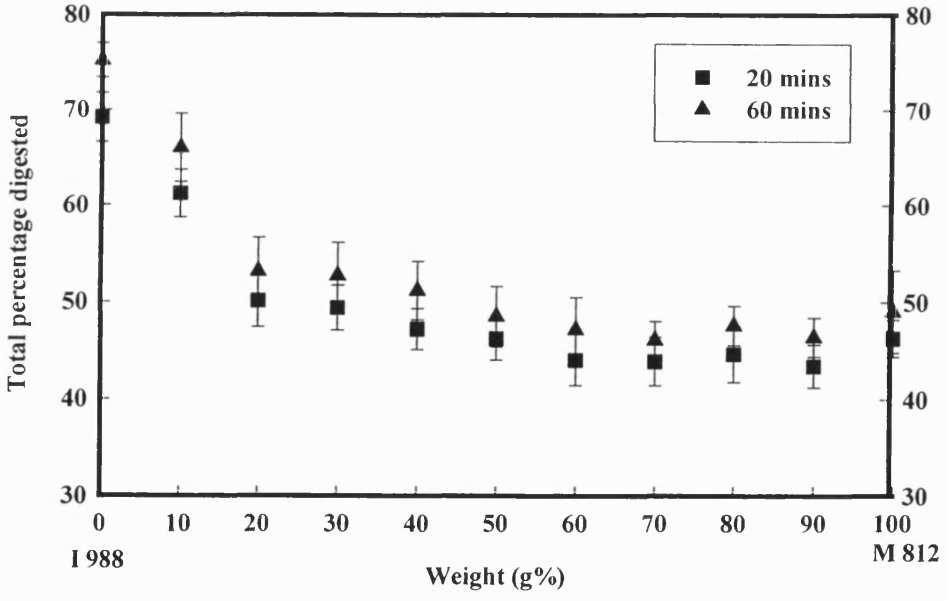


Figure 5.12 Profile lipolysis of SEDDS formulations under standard pH-stat conditions

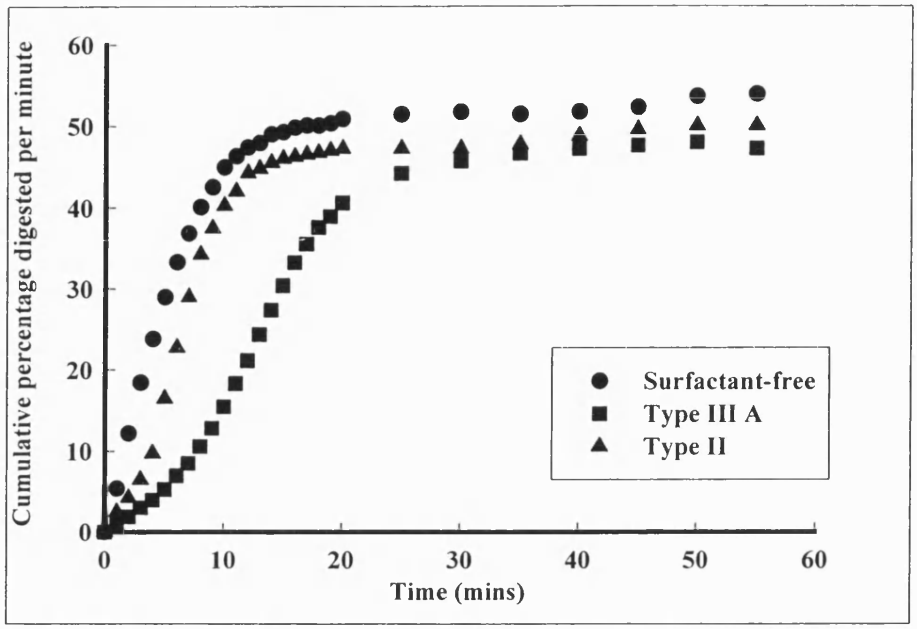
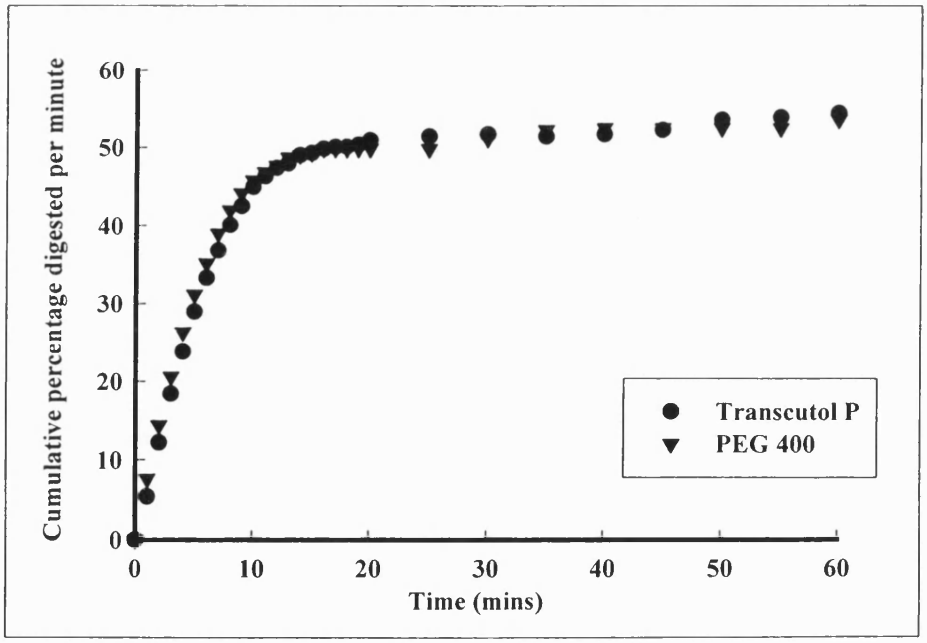


Figure 5.13 Profile lipolysis of surfactant-free formulations in the presence of different hydrophilic cosolvent under standard pH-stat conditions



Chapter 6

Phase separation of lipid formulations following their dispersion in simulated intestinal fluid.

6.1 Introduction

The fate of lipids in upper small intestine contents following a lipid-rich meal in humans has been studied extensively by Borgström and co-workers (1962) (reviewed by Staggers *et al*, 1990). Although much work has been done to explore the various biochemical steps in lipid digestion [reviewed by Borgström (1977b), Carey (1983); and Hernell *et al* (1990)], the nature of the physical-chemical events involved still not completely understood. Hofmann and Borgström (1963, 1964) reported a classical hypothesis that intestinal lipids, during fat digestion, are partitioned between two or three physical states in the duodenal contents: an oily rich or emulsion portion, rich in TGs and DGs; a dilute, aqueous mixed micellar phase composed of bile salts and lipolytic products, and a precipitated "pellet" (Borgström, 1985; Hernell *et al*, 1990). The absorption of dietary lipid takes place from a micellar solution containing chiefly fatty acid and 2-MG. Later, Patton and Carey (1979) examined fat digestion using triolein as the substrate, *in vitro* under the light microscope, in the presence of bile salts and pancreatic lipase and colipase. They saw the sequential formation of two visible lipolytic phases: a crystalline phase followed by a viscous isotropic phase. A crystalline phase was identified as containing birefringent calcium soaps and ionised fatty acid, whereas viscous isotropic phase was composed mainly of MGs and protonated fatty acids (1:1) (Patton *et al*, 1981). They suggested that the viscous isotropic phase formed myelin figures in unsaturated bile salts solution and eventually dissolved into a clear micellar solution. In bile saturated with lipids, neither phase disappeared completely. When centrifuged, the first crystalline phase sedimented as a pellet. The upper (floating) oil phase contained mostly unhydrolyzed TG and some DGs; the aqueous phase was turbid and contained mainly MG and fatty acids with small amounts of di- and tri-glycerides. The density of the viscous

isotropic phase after centrifugation seen on the slide was similar to that of the micellar phase. Therefore, the micellar phase in most instances may be a two-phase system and the rate of formation relative to the rate of solubilization into the micellar phase will determine the phase relationships occurring during *in vivo* digestion (reviewed by Borgström, 1985).

In 1990, Staggers *et al* updated the intestinal hypothesis through the definition of the complete condensed phase diagram of one system for typical physiological conditions. They also have determined the influence of several physical-chemical variables upon the phase boundaries of physical-chemical compositions of a two-phase zone. In healthy adult humans, duodenal contents were collected and aspirated following the feeding of a triacylglycerol-rich meal. The aspirates were collected during established lipid digestion and absorption into a "cocktail" of chemical inhibitors that rapidly inhibited *ex vivo* lipolysis. Ultracentrifugation showed that the lipids separated into a floating oil layer, several oil layers, several interfacial layers, a "clear" or turbid "subphase" and a precipitated "pellet" (Hernell *et al*, 1990). They identified the floating layer as oil-in-water emulsion particles with cores of triacylglycerol (TG), diglycerols (DG) and choesteryl esters (CE) emulsified with a surface coat of partially ionised fatty acids (FA), MGs (MG), diacylphosphatidylcholine (PL), and bile salts (BS). The interfacial layers exhibited lamellar liquid crystalline structure with similar emulsion particles dispersed among emulsifier. The subphases were identified as saturated mixed micelles composed of BS, FA acid-soap, MG, PL, cholesterol (Ch), and traces of DG and TG coexisted with unilamellar liquid-crystalline vesicles composed of the same lipids. Precipitated pellets were composed principally of emulsifying lipids, with smaller amounts of crystalline calcium soaps and BS (Hernell *et al*, 1990).

In the work described in this chapter, the physical chemistry of lipid excipients, SEDDS formulations and mixtures of mono-, di-, and tri-glycerides *in vitro* under standard pH-stat condition has been studied systematically. In the model lipid system used, phases were identified after ultracentrifugation and compared with the model

mentioned by Hernell and Staggers (1990). The fate of hydrophobic drugs was determined in each phase using HPLC method.

6.2 Materials and methods

6.2.1 Materials

In addition to the materials mentioned in chapters 2 and 4, the following materials were used:

- Methanolic cocktail solution:

Name	Concentration	Cas. #	Manufacture
Diisopropylfluorophosphate	50 mM	D 0879	Sigma
Diethyl (*p*-nitrophenyl) phosphate	50 mM	D 9286	Sigma
Acetophenone	50 mM	A1, 070-1	Aldrich
Phenyl-boronic acid	250 mM	P2, 000-9	Aldrich

The previous materials were diluted in methanol (99% v/v) in concentration not more than 2% w/w (Hernell *et al*, 1990).

- Drugs:

- The same drugs used in Chap. 2
- Drugs used as internal standard for HPLC analysis are the following:

Name	Concentration	Cas. #	Manufacture
Corticosterone	0.000682 g %	C 2505	Sigma
Dexamethasone	0.000264 g %	D 1756	Sigma
Testosterone	0.000792 g %	T 1500	Sigma
Hydrocortisone	0.000653 g %	H 4001	Sigma

- Phosphate buffer pH 6.5

		Mwt	Cas. #	Concentration	Used *	Manufacture
A	Potassium phosphate Monobasic, KH_2PO_2	136.1	P 5379	9.079 g/ L *i.e.* 6.671mM	778 ml	Sigma
B	Sodium phosphate Dibasic, Na_2HPO4	142	S 7907	11.87 g/ L *i.e.* 8.36mM	338 ml	Sigma
C	Hexadecyltrimethyl ammonium Bromide (Cetrimide per USP)	364.5	H 5882	1.68 g / L *i.e.* 0.461 mM	1.68 g / L	Sigma

Phosphate buffer (pH 6.5) prepared by mixing 350ml of mixture of A and B solutions, used in proportions shown in the accompanying Table, made up to 1L with methanol (99 % v/v), followed by adding cetrimide.

- Composition of lipid tested:

Lipid excipients and formulations tested are summarized in Table 6.1.

6.2.2 Methods

6.2.2.1 Visual inspection

A visual test of mixtures of mono-di-, and tri-glycerides (Imwitor 988®) and medium chain fatty acid oil (Miglyol 812®) with various aqueous phases or simulated intestinal fluids was carried out as shown in Tables 6.2 and 6.3. The excipients or formulations were introduced into 100 ml volumetric flask of solutions shown in Tables 6.2 and 6.3 at 25°C and the content were mixed vigorously for 5 min with a magnetic stirrer. The tendency was for phases to separate spontaneously. The identification of phases present was easier when mixtures were left for at least one hour. Photographs were taken using a digital camera to help identify and compare the phases. All experiments were repeated twice, with similar observations being made between repeats (Kummuru *et al*, 2001).

	Butyl paraben		Methyl paraben		Hydrocortisone		Testosterone	
	5min	30 min	5min	30 min	5min	30 min	5min	30 min
MCT (Miglyol 812®)	✔	✔	✔	✔	✔	✔		✔
LCT (Corn oil)	✔	✔	✔	✔	✔	✔	✔	
MCT (Miglyol 812®) without Ca	✔	✔			✔	✔		
LCT (Corn oil) without Ca	✔	✔			✔	✔		
Mixed glycerides Imwitor 988®	✔	✔		✔	✔	✔		✔
Surfactant- Free M 812®+ I 988®+ PG 30% + 50 % + 20%	✔	✔		✔	✔	✔		✔
Surfactant- Free without Ca	✔	✔			✔	✔		
Type II M 812® + TTO® 40 % + 60 %	✔	✔		✔	✔	✔		✔
Type III A I 988® + M 812® + CRH 40® 35 % + 35 % + 30 %	✔	✔		✔	✔	✔		✔
Type III B I 988® + M 812® + CRH 40® + PG (9 : 1)35% + 35% + 30%	✔	✔		✔	✔	✔		✔
I 988® + M 812® 50 % + 30 %	✔	✔		✔	✔	✔		✔
I 988® + M 812® 50 % + 50 %	✔	✔		✔	✔	✔		✔
I 988® + M 812® 30 % + 70 %	✔	✔		✔	✔	✔		✔
I 988® + M 812® 70 % + 30 %	✔	✔		✔	✔	✔		✔

Table 6.1 Lipid excipients and formulations containing lipophilic drugs used in the investigation (✔ indicates that the experiments were done in terms of lipolysis and analyzed using the HPLC).

Solutions	Imwitor 988®	Miglyol 812®
H_2O	✔	✔
Trizma-buffer® (pH 7.0)	✔	✔
Trizma-buffer® (pH 7.0) + BS (15 mM)	✔	✔
Standard pH-stat solution (non-pure LC)	✔	✔
Standard pH-stat solution (pure LC)	✔	✔

Table 6.2 Mixture of glycerides and medium chain oil in different solutions were examined for visual phase separation.

Imwitor 988®		Miglyol 812®	Standard pH-stat solution
2 g	+	zero	✔
2 g	+	0.4 g	✔
2 g	+	0.8 g	✔
2 g	+	1.2 g	✔

Table 6.3 Mixture of glycerides and medium chain oils were mixed in standard pH-stat solution for visual phase separation.

6.2.2.2 In vitro lipolysis

A series of lipid excipients and self-emulsifying systems (4 g in 100 ml) as shown in Table 6.1 were dispersed in standard pH-stat solution and subjected to lipolysis (Chapter 4, section 4.4.6.4.2) under standard pH-stat conditions for studies of phase behaviour after lipolysis. At 30 min, following the addition of the lipase to the simulated intestinal fluid, <2%v/v (1 ml by Gilson pipette) of methanolic solutions of a "cocktail" of lipase inhibitors was added to quench the reaction (material, 6.2.1).

6.2.2.3 Separation and collection of phases

To evaluate the efficiency of speed and duration of centrifugation, different samples were run at the same speed, 104630g, for different times. Later, all mixtures were ultracentrifuged at 104630g for 30 min at 37°C by using ultra clear tubes

(38×102 mm), capacity 94 ml, in a SW–70.1 swinging bucket rotor (Model L8 – 70M ultracentrifuge, Beckman Instruments, Palo ALTO, CA).

The separated layers were aspirated from the ultracentrifuge tube into snap-cap microtubes (1.5 ml, 40 H×11 mm OD, Sigma-Aldrich, UK) in the manner described by Hernell *et al* (1990). The oily layer was collected first with a MedSaver disposable syringe (1 ml with 25 gauge, 5/8 – in needle, Sigma-Aldrich, UK). After the oily layer was removed from the top, portions of the interface were withdrawn using MedSaver disposable syringes, placing the syringes at the side of the tube so as to minimize the contamination between phases. Only very small volumes (5–25 µl) of interface could be withdrawn before being contaminated with the micellar phase. A syringe with a stainless steel syringe needle (2 in length, 14 gauge) was then used to puncture the side of the tube at the level of the subphase (micellar phase). Thereafter, sufficient amount was removed, another syringe inserted to a deeper level to withdraw what is referred to as the swollen micellar phase. Finally, the pelleted or precipitate fraction was obtained first by removing all the supernatant material from the tube and scraping a spatula (Hernell *et al*, 1990). All the snap-cap microtubes were kept in the fridge for HPLC analysis.

6.2.2.4 Assay method for lipophilic drugs

Table 6.4 summarized the assay parameters for hydrocortisone, testosterone, butyl paraben, and methyl paraben. They were essentially the same, except for minor variations in the mobile phases and internal standards used in the chromatographic analysis (Charman *et al*, 1986). A 200 µl sample of each was diluted in the presence of internal standard (IS) (material, 6.2.1) with the specified solvent (Table 6.4). A 100 µl diluted sample preparation was subjected to HPLC analysis under the following conditions; pump model 110A, UV detector model 153 (Beckman, Berkeley, CA 94710, USA), 25cm ×4.6mm Hichrom C18 BDS and the guard column 5 µm S5ODS2- 10C5. The method of internal standards was used to convert

Drugs	Log P	Mwt	λ max	Mobile phase	Internal standard	RT of IS	RT of drug	Injection volume	Solvent	Flow rate
Hydrocortisone	1.53	362.5	242	MeoH: H_2O 55%: 45%	Cotricosteroid	12 min	9.2 min	100 mM	MeoH 99 % v/v	1 ml/min
Testosterone	3.3	288.4	240	ACE: H_2O 55 %: 45%	Dexamethasone	4.19 min	8.83 min	100 mM	MeoH 99 % v/v	1 ml/min
Butyl paraben	3.24	194.2	252	Phs buffer: MeoH 35%: 65%	Testosterone	13 - 14 min	11 min	100 mM	ACE 99 % v/v	1 ml/min
Methyl paraben	1.66	152.1	231	ACE: H_2O 27.5%: 72.5%	Hydrocortisone	12.6 min	7.76 min	100 mM	ACE 99 % v/v	1 ml/min

Table 6.4 Parameters used for the HPLC analysis.

the measured peak heights of the drug and the internal standard to concentration of the drug. Values are reported as mean ± SD and the data were considered statistically significant at $p < 0.05$ (Kummuru *et al*, 2001). Calibration curves for standard solutions were done with a correlation coefficient in all cases (R^2 values of 0.999 were typical) and summarized in Appendix 3 (Bakatselou *et al*, 1991).

6.3 Results

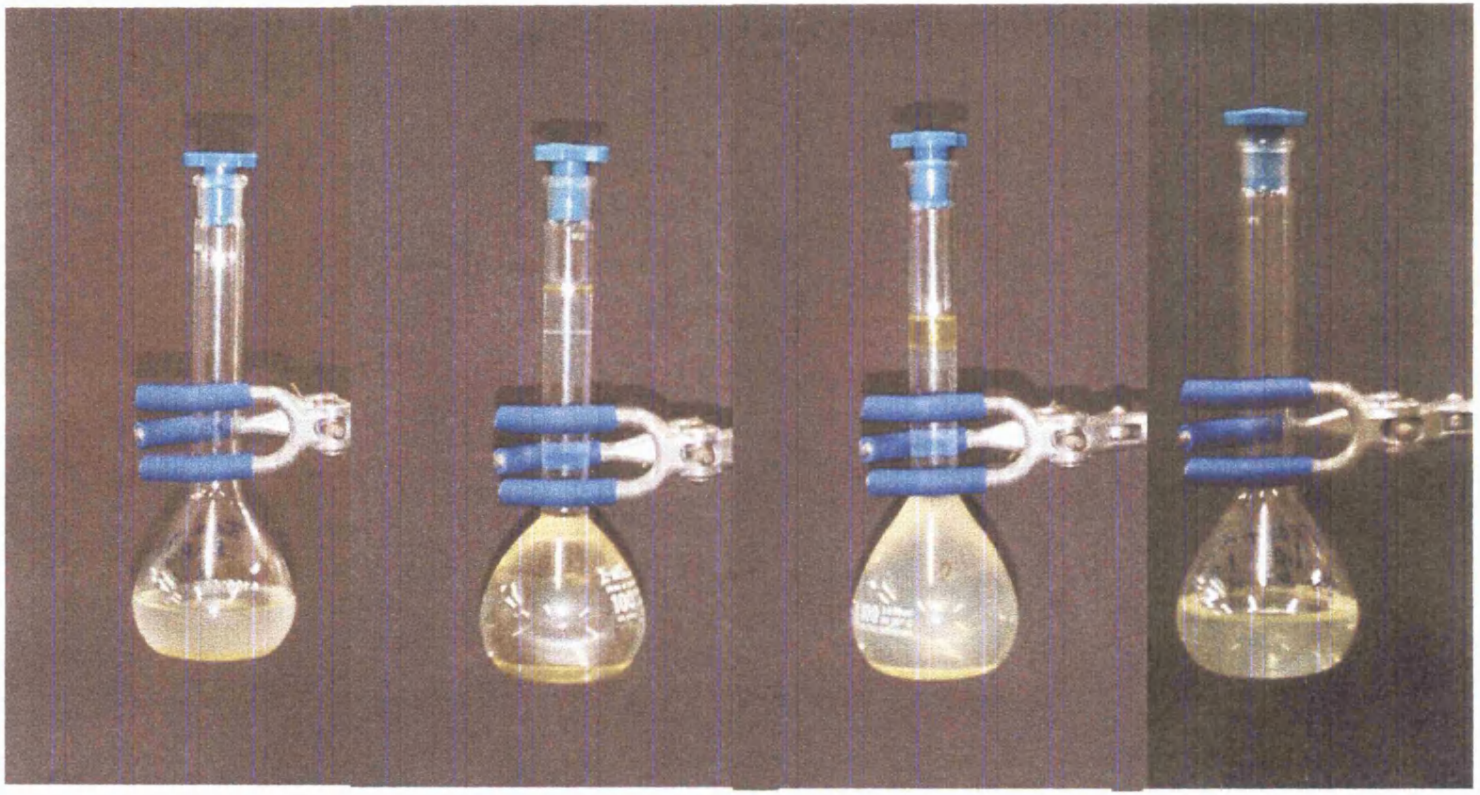
6.3.1 Visual inspection of mono-, di- and tri-glycerides behaviour in water, tris-maleate buffer (pH 7.0) and standard pH-stat solution at ambient temperature.

Any understanding of the complex process of fat absorption requires clarification of the physico-chemical state of lipids in intestinal contents before and/or after digestion (Hofmann, 1963). Naturally, ingested TGs are hydrolysed by pancreatic lipase mainly to fatty acids and MGs, and these polar lipids are solubilized in bile salt micelles to form an isotropic micellar solution. All formulations dispersed to form emulsions in pure water but their behaviour in standard pH-stat solution, was dependent on MGs (Dressman *et al*, 1998). Therefore, some experiments were performed by mixing different weights of mixed glycerides (Imwitor 988®) and medium chain oil (Miglyol 812®) as shown in Table 6.3 in standard pH-stat solution at ambient temperature and then left to equilibrate. The visual test provided a measure of the apparent spontaneity of phase separation of lipid excipients in 100 ml standard pH-stat solution. In the present study, when sufficient MG was present >60%, as in Imwitor 988® demulsification and phase separation was observed, which was dependent on the presence of phospholipid and resulted in sedimentation of what was believed to be a phase rich in MGs and water, the "swollen gel phase" as illustrated in Figure. 6.1. The addition of TGs >30%, as in Miglyol 812® stabilized the formation of "mixed micelles", which remained in a finely dispersed state. The tendency of MG to sediment was spontaneous and the more the solution was allowed

to stand, the more clearly the phases were distinguished (Figure. 6.1). The addition of the medium chain oil (mixture of $C_8 \approx 60\%$ and $C_{10} \approx 40\%$ of DGs) gradually increased the micellar phase, containing MGs and some DGs, and decreased the extent of sedimentation of MGs ($C_8 \approx 90$ of mixture of MG and DG). At >30% medium chain oil and >50% mixture of mono-, di- and TGs, only two phases were seen; the oily phase at the top containing the TG and DG, and the micellar phase slightly turbid solution containing MGs and the free fatty acids. In other experiments, adding mixtures of glycerides (Imwitor 988®) either to water or tris-maleate buffer (pH 7.0), made no difference in terms of solubilization; both caused sedimentation of MG in a viscous gel like phase. Adding bile salt and phospholipid (lecithin either pure or non-pure) to tris-maleate buffer exhibited the same behaviour as when the tris-maleate buffer was used alone (Table 6.2). Adding medium chain oil (Miglyol 812®) to water, tris-maleate buffer (pH 7.0) and standard pH-stat solution gave two phases. The oily phase is at the top and the rest is clear micellar phase. This is explained by the presence of mixed micelles comprising lecithin and bile salt was capable of solubilizing TG and DG to an appreciable extent under similar conditions to that of the intestine (pH 7.0) (Smith *et al*, 1976).

In 1963, Hofmann studied the behaviour and solubility of MGs in dilute and micellar bile salts solution. He mentioned that 1-monolaurin (C_{12}) behaves as an amphiphile in dilute bile solution at 37°C. Its saturation ratio (*i.e.* micellar MGs/ micellar bile salt) is considerably higher than that of 1-monomyristin(C_{14}). The excess forms a viscous, slightly turbid phase which slowly settles; the phase is not birefringent. 1-monodecanoin (C_{10}) and 1-monooctanion (C_8) behave similarly to 1-monolaurin. But their micellar solubilities are much higher (Hofmann *et al*, 1963).

One early hypothesis suggested that solutions of amphiphilic MGs and non-polar MGs become clear when equilibrated simultaneously with bile salts solution. Initially, only the amphiphilic MG can be solubilized to any extent. As its concentration in the micelle is increased, the resulting micelle can dissolve progressively more of the high melting point MG and remain liquid. However, if the



[1]Imwitor 988® (2g) in simulated intestinal pH-stat fluid (50ml).

[2]Imwitor 988® (2g) + Miglyol 812® (0.4g) in simulated intestinal pH-stat fluid (100ml).

[3]Imwitor 988® (2g) + Miglyol 812® (0.8g) in simulated intestinal pH-stat fluid (100ml).

[4]Imwitor 988® (2g) + Miglyol 812® (1.2g) in simulated intestinal pH-stat fluid (50ml).

Figure 6.1 Represents the fate of MG in simulated intestinal pH-stat fluid. When sufficient monoglycerides were present (> 60 %, Imwitor 988®), we observed demulsification and phase separation[1], which was dependent on the presence of phospholipid and resulted in sedimentation of what was believe to be a phase rich in monoglycerides and water "swollen micelle". The addition of triglycerides (>30%, Miglyol 812®) stabilized the formation of mixed micelles, which remained in a finely dispersed state as seen in photographs 2, 3 and 4.

micelle contains large quantities of low melting point MG, at that point the solubilization of a MG becomes competitive. As non-polar MG is added to an aqueous bile salt solution, there is usually a change from an isotropic micellar solution to a liquid crystalline state. When excess MG is added to bile salts solution, a turbid phase appears which contains bile salt, MG, and water, and may form birefringence (Lawrence, 1961). The MGs may behave as an amphiphilic or non-polar solute in bile salts solution depending on the experimental temperature. Body temperature is constant 37°C. Therefore, the normal products of pancreatic lipolysis are mainly 2-monoglycerides and fatty acids. The 2-monoglycerides will generally be unsaturated and, therefore, amphiphilic. Amphiphilic MGs and fatty acids are competitively solubilized by bile-salt solutions. Therefore, the intestinal content after a meal may be considered as amphiphilic, if the MG and fatty acids are considered as a single phase. They will be partitioned between the bile salt micelles and the emulsified oil phase; the oil phase containing most of the DG and TGs present in the intestinal contents (Borgström, 1985).

6.3.2 Phases behaviour of lipid excipients and formulations under standard pH-stat conditions

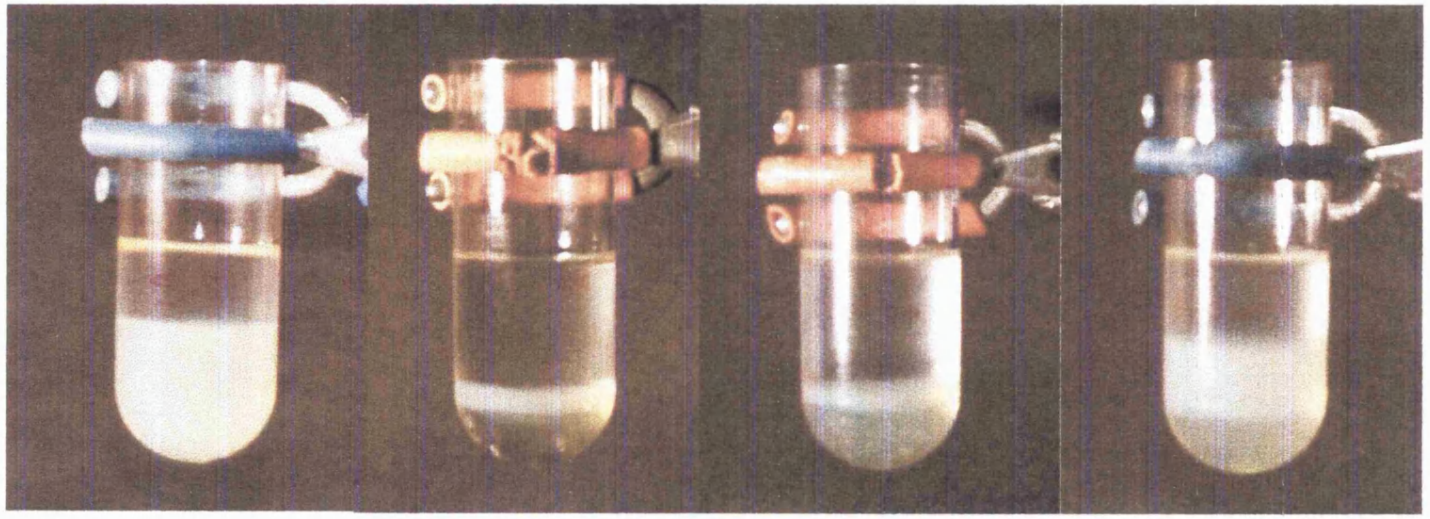
The physical and chemical nature of the dispersed hydrolytic products of lipid excipients and formulations were studied under standard pH-stat conditions in 100ml standard pH-stat solution. The following lipid excipients and formulations containing hydrophobic drugs (hydrocortisone, testosterone, butyl paraben, and methyl paraben) were studied after *in vitro* lipolysis under standard pH-stat conditions, as described in chapter 4. Because the hydrolysis of lipid involves several complex steps of phase behaviour, the lipolysis process was stopped after 30 min following addition of pancreatin lipase to the simulated intestinal fluid, by adding a methanolic solution of a cocktail of enzyme inhibitor, facilitating the possibility of identifying the phases by ultracentrifugation. Lipolysis was stopped at 30 min because at this stage at least 50% of the lipid was digested (chapter 4) which helped the clear detection of the phases present.

A cocktail of enzyme inhibitors was used because no single inhibitor was completely effective in inhibiting colipase-dependent pancreatic lipase; hence, a combination of diethyl (4-nitrophenyl) phosphate and phenylboronic to inhibit lipase, diisopropylfluorophosphate to inhibit carboxyl ester hydrolase Cholesteryl esterase, and acetophenone to inhibit pancreatic phospholipase A2, were employed (Hernell *et al*, 1990). Earlier studies by Hofmann and Borgström (1964) showed that heating intestinal content at 60°C for 10 min completely inhibited lipase activity. They did control experiments showing that such heat inactivation or subsequent maintenance at 70°C for 1 hour did not alter the glyceride or fatty acid composition of intestinal content. Later, experiments were performed showing increased release of FA from heated intestinal contents. The source was predominantly TG (Porter *et al*, 1971). Heating would enhance lipase activity, and TG would be hydrolyzed rapidly.

Another source of fatty acid was lecithin. In human intestinal contents, there is a pancreatic phospholipase which hydrolyzes lecithin to 1-acyl lysolecithin. The lecithinase is stable at 70°C. Therefore, even if pancreatic lipase as instantaneously inactivated in a sample of intestinal contents, there would be perturbations in phospholipid composition which might affect micellar size and alter lipid solubilization (Porter *et al*, 1971).

Centrifugation time had some impact on the apparent phase behaviour but generally only led to differences in the relative phase volumes, especially of the subphases and the interfacial phases. The greater the centrifugal time, the closer was the micellar phase boundary to that of the subphase, indicating a relative increase in micelle-to-vesicle ratios (Staggers *et al*, 1990). Figure 6.2 shows that with increasing time of centrifugation the white layer increased and sedimented leading to interference with the viscous gel-like layer at the bottom of the tube.30min centrifugation at 104630

The centrifuged samples were distinguished into oils, interfaces, sub-phase, gel-like viscous layer and precipitates (pellets); these are arbitrary distinctions that follow literature usage (Carey *et al*, 1983). Although the phases were ultracentrifuged, they



Surfactant- free formulation (4g) + 1% w/v hydrocortisone digested for 20 min under standard pH-stat conditions.	Surfactant- free formulation (4g) + 1% w/v hydrocortisone digested for 20 min under standard pH-stat conditions and centrifuged at 104630 g for 10 mins.	Surfactant- free formulation (4g) + 1% w/v hydrocortisone digested for 20 min under standard pH-stat conditions and centrifuged at 104630 g for 20 mins.	Surfactant- free formulation (4g) + 1% w/v hydrocortisone digested for 20 min under standard pH-stat conditions and centrifuged at 104630 g for 30 mins.

Figure 6.2 The effect of centrifugation time on the phase separation. There were generally difference on the relative phase volume especially the subphase and the interface. The greater the centrifugal time, the closer to the micellar phase (subphase) boundary to the interface, indicating a relative increase in micelle-to-vesicle ratios. Therefore, 30mins centrifugation at 104630 g was chosen as a standard for most samples so that direct comparison could be made.

were not true homogenous phases, due to collection difficulties as described in methods (6.2.2.3) (Hernell *et al*, 1990).

The phases of lipid excipients and formulations were described below under the conditions mentioned before (section 6.2.2.2 and 6.2.2.3):

- **Long chain TG (Corn oil)**
  Oily layer at the top (6–8ml) followed by milky, thick solution, believed to be micellar phase (<88ml).

- **Medium chain TG (Miglyol 812®)**
  Oily layer at the top (4–6ml) followed by clear to slightly turbid solution, (<80ml) with some precipitates at the bottom of the tube.

- **Mixed mono-, di-, and tri-glycerides (Imwitor 988®)**Oily layer at the top (2–4ml) followed by a clear micellar phase (<38ml) and then viscous gely structure at the bottom (<50ml).

- **Type II (Miglyol 812®: Tagat TO®)**
  (40%: 60%)

  Micellar phase (subphase), white to slightly turbid was identified (<55%) followed by condensed thick layer at the bottom full of some pellets (>35%). An interface can be identified.

- **Type III A (Imwitor 988® + Miglyol 812® + CRH 40®)**
  (35%+ 35% + 30%)

  The solution was a clear micellar solution (subphase) (<90%) with some pellet at the bottom of the tube. The top of the solution (interface) (5–8ml) was more transparent than the micellar phase.

- **Type III B ({Imwitor 988® + Miglyol 812® / 9:1 }+ CRH 40® + PG) (35%+ 35% + 30%)**
  The micellar solution (subphase) was clear (100ml).

- **Surfactant-free formulation (Miglyol 812® + Imwitor 988® + PG) (30%+50%+ 20%)**

  At the top, interface (2–3ml) was slightly clear than the rest of the micellar solution (<45%) followed with subphase (micellar phase) (>50%).

- **Mixture of mono-, di-, and tri-glycerides (Imwitor 988®) and MCT (Miglyol 812®) (50% + 50%)**

  Slightly oily layer at the top (3–4 ml) followed by a slightly interface (1–2 ml). The micellar solution (subphase) was clear (>50ml) followed by a viscous gel- like phase (<30 %).

- **Mixture of mono-, di-, and tri-glycerides (Imwitor 988®) and MCT (Miglyol 812®) (30% + 70%)**

  Oil layer was at the top (5–7 ml) followed by slightly turbid thick solution with pellet at the bottom (>90 ml).

- **Mixture of mono-, di-, and tri-glycerides (Imwitor 988®) and MCT (Miglyol 812®) (70% + 30%)**

  Slightly oily layer at the top (1–2 ml) followed by interface (1–2 ml). Micellar solution (subphase) was clear transparent (<20ml) followed by viscous gel-like phase (swollen micellar) (>70 ml).

- **Mixture of mono-, di-, and tri-glycerides (Imwitor 988®) and MCT (Miglyol 812®) (50% + 30%)**

  Slightly oily layer at the top (1–2 ml) followed by interface (8–12ml). Micellar solution (subphase) was clear transparent (<70ml).

6.3.3 Phase analysis of hydrophobic drugs in lipid excipients and formulations under standard pH-stat conditions.

The drug uptake in each lipid excipient and/or SEDDS formulations and mixtures of glycerides was analysed by HPLC after ultracentrifugation at 106430 g for 30 min. The concentration g %w/v of each drug in each phase of lipid excipients, SEDDS formulations, and mixture of glycerides were summarized in Appendix 3. It can be seen from the data that there was no significant difference in the distribution of the drug in each phase after 5 min or 30 min ultracentrifugation as shown in Figures 6.6-6.16. Therefore, the drug concentration distributed in the phases was discussed based on ultracentrifugation 106430g for 30 min.

- *Lipid excipients*

As mentioned earlier a series of *in vitro* lipolysis experiments using 4 g of LCT (Corn oil®), MCT (Miglyol 812®), and mixture of mono-, di-, and tri-glycerides (Imwitor 988®) under standard pH-stat conditions was performed and quenched 30min after addition of pancreatin enzyme by adding cocktail of pancreatin enzyme inhibitor.

Following ultracentrifugation of LCT, two phases were observed, an oily phase at the top, followed by milky turbid phase crude emulsion as shown in Figure 6.3. The presence of a similar white pasty phase has been noted previously in potassium oleate systems in the absence of bile salt over a pH range of approximately 7.0-8.0, which was thought to be a metastable cubic phase water in oil lattice (Cistola *et al,* 1988). The oily phase in these studies comprised primarily of triglycerides and diglycerides with small amounts of monoglyceride and fatty acid. The white precipitated pellet contained fatty acid. In the presence of fasted bile salt concentrations, a larger proportion of the fatty acid and monoglyceride was distributed into the aqueous phase, but the relatively high lipid/bile salt mole ratios resulted in the production of a turbid aqueous phase, presumably indicating the presence of incompletely micellar-solubilized lipids, and the coexistence of micellar, liquid crystalline and liposomal or

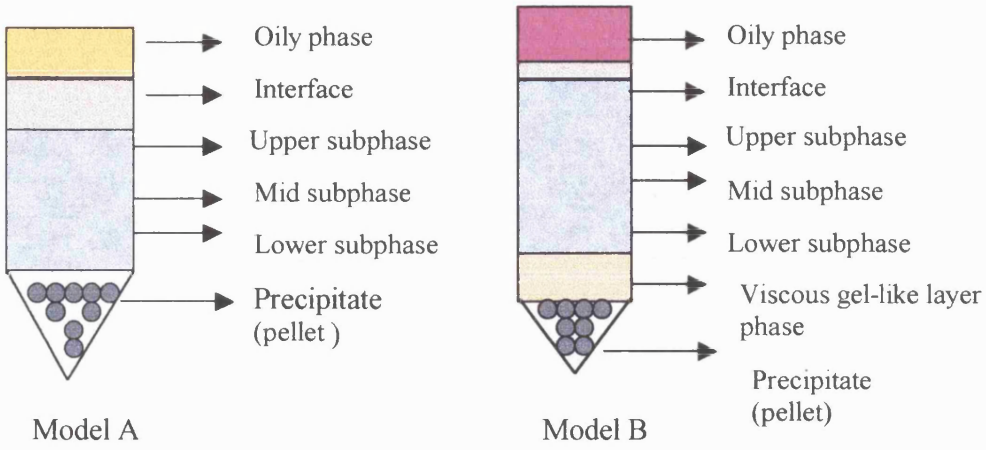
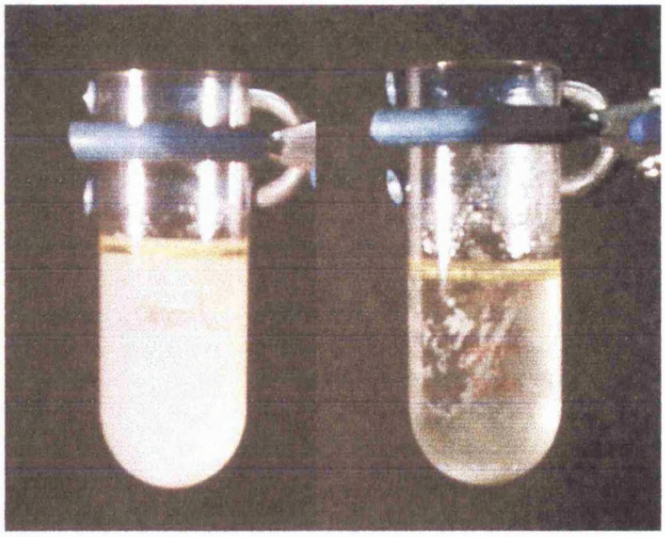


Figure 6.3a Schematic model of a typical sample of distal duodenal contents following ultracentrifugation as described by Hernell *et al* (1990), model A. Model B a representative model of the *in vitro* lipolysis under standard pH-stat conditions following ultracentrifugation throughout the research.



LCT (Corn oil) MCT (Miglyol 812®)

Figure 6.3b Representative example of the fate of LCT (Corn oil) and MCT (Miglyol 812®) under standard pH-stat conditions after ultracentrifugation 106430 g for 30 mins in simulated intestinal pH-stat solution.

vesicular structures (Sek *et al*, 2002). At higher bile salt level *i.e.* fed state the aqueous phase become less turbid, reflecting improved micellar solubilization.

For LCT (Corn oil®), the distribution of the drugs in each phase was similar. More than 80% of the drug was in the milky turbid phase. The reminder was in the oily phase. The mass balance where the concentration of the drug in 4 g of lipid excipients, SEDDS formulation and mixture of glycerides in 100ml standard pH-stat solution was different for each drug in each lipid excipients and/or lipid formulations. Therefore, the concentration of drug in each phase was different. In Figures 6.5 and 6.6 respectively showed that the concentration of butyl paraben (BP) (Log *P* 3.3, Mwt = 194.2) in the milky turbid phase was 0.081 g%, while testosterone (Log *P* 3.7, Mwt = 288.4) was 0.0071g%. A similar order can be seen with drugs with log *P* <2. For example, Figures 6.7 and 6.8 respectively showed that hydrocortisone concentration was 0.0261 g % and methyl paraben concentration was 0.0808 g%. The molecular weight of the drug and the partition coefficient play a major role in the distribution of the drug. The concentration of the BP in the oily layer was 0.079 g% and MP was 0.0425 g% w/v as shown in Figures 6.5 and 6.8 respectively. The solubility of steroids was lower than hydroxy benzoate derivative because of the high molecular weight 0.0005 g% for hydrocortisone and 0.004 g% for testosterone.

In the case of MCT (Miglyol 812®) the distribution of the drug was different from the corresponding LCT system because of the presence of an oily phase at the top followed by slightly turbid phase micellar or subphase and some pelleted material at the bottom as shown in Figure 6.3. Recently, Sek *et al* (2002) mentioned that compared with long chain lipolytic products, a significantly higher proportion of the medium chain lipolytic products dispersed into the aqueous phase, approximately 80%. The aqueous phase comprising approximately 100mM fatty acid and 20 mM MG, obtained after MCT digestion in the absence of bile salt was turbid, suggesting the presence of larger colloidal structures, whereas, at increasing bile salt concentrations, the turbidity was reduced, indicating improved solubilization of the lipolytic products into mixed micelle (Sek *et al*, 2002). The concentration of BP in

micellar phase was 0.096 g%, while the testosterone was 0.0164 g% as shown in Figures 6.5 and 6.6 respectively. A similar observation was noticed for hydrocortisone 0.0087 g% and MP 0.012 g%, as shown in Figures 6.7 and 6.8 respectively. The percentage of drug dissolved in the oily phase of MCT was higher than in LCT. This could be explained by the volume of the oil phase which being higher in LCT than in MCT after ultracentrifugation; therefore, the drug would be more diluted. Besides, the original solubility of each drug in MCT was much higher than in LCT (Chap. 2). For example, the concentration of the BP in the oily phase of MCT was 0.093 g% while in the oily phase of LCT was 0.0791 g% as shown in Figure 6.5. MP concentration was 0.066g% in the oily phase of MCT and 0.0425 g% in the oily phase of LCT as shown in Figure. 6.8. In the presence of LCT (corn oil), more than >65% w/v of the drug remained in the aqueous micellar phase. On the contrary, MCT (Miglyol 812®) was not capable of maintaining more than 30%w/v of the drug in solution. After ultracentrifugation lumps was noticed on the wall of the centrifuge. The precipitate phase was thought to be calcium soaps. Therefore, experiments were done in the absence of calcium using MCT as a substrate. Surprisingly, the precipitate was still found after centrifugation as well as the lumps. As a result, these lumps were thought to be a mixture of undigested MG and DG precipitated due to an excess centrifugation.

In the case of mixtures of mono-, di-, and tri-glycerides (Imwitor 988®), four phases were distinguished; an oily phase at the top followed by the micellar phase, followed by the viscous gel-like structure believed to be monoglycerides and some pellet at the bottom. In contrast to MCT digests, the aqueous phase (comprising 100mM C_8/C_{10} fatty acid only) obtained after C_8/C_{10} MG/DG digestion was clear even in the absence of bile salt (Sek *et al*, 2002). The turbidity present in the MCT digests results from the presence of MG and is consistent with the relatively low aqueous solubility of monodecanoin in the absence of bile salt (approximately 4mM in sodium phosphate buffer), but increased solubility in the presence of bile salt (11mM in 4mM NaTDC) (Hofmann, 1963). The clarity of the aqueous phase obtained after digestion mixed

mono-, di-, and tri-glycerides suggested that substantial quantities of lamellar phase were not formed or were pelleted out during centrifugation under these circumstances. This may reflect the higher proportion of C_8 fatty acid resulting from the digestion of Capmul MCM® or Imwitor 988® *e.g.* Capmul MCM glycerides contain 17% C_6, 80% C_8 and 18.3% C_{10} fatty acids when compared with Miglyol 812® comprising 0.23% C_6, 59.5% C_8, 39.8% C_{10}, 0.24% C_{12}.

In comparison with long chain lipid digestion, the higher rate and extent of digestion and greater aqueous phase distribution of medium chain lipolytic products may be attributed to several factors including solubility, hydrophilicity and phase behaviour. Medium chain fatty acids are ionised to a greater extent (apparent pK_a 6.8) than long chain fatty acids (pK_a 8-8.5) at physiological pH, therefore increasing solubility and amphilicity and enhancing the potential for formation into bile salt micellar system (Cistola *et al*, 1988). Furthermore, the aqueous solubility of medium chain digestion products is significantly higher than that of long chain digestion products. For example, the aqueous solubility of decanoic acid is approximately 25μM whereas the solubility of oleic acid is approximately 1 μM. Partial ionisation of medium chain fatty acids may also lead to the production of stable fatty acid/ fatty acid soap lamellar phases (Cistola *et al*, 1988), the formation which may encourage aqueous dispersion of medium chain lipid digestion products even in the absence of bile salt. Medium chain lipolytic products therefore rapidly dissociate from the digesting interface forming either a simple solution or colloidal dispersion, or may precipitate as soaps. This is in contrast to long chain systems where removal of lipolytic products is limited by the concentration of the bile salt, and the solubilization capacity of the digestion media.

Surprisingly, at least 70% of the drug was dissolved in the viscous gel-like phase. The fate of the drug could be precipitation because MG and FA could be absorbed through passive diffusion leaving the drug to precipitate. Therefore, having more than 50% of MG in formulation is undesirable. Less than 20% of the drug was dissolved in micellar phase as shown in Figures 6.5-6.8. The solubility of BP in the

viscous gel-like phase 0.151 g% and for MP was 0.362 g%. However, in the micellar phase of mixed mono-, di- and tri-glycerides, the solubility of BP was 0.016g% and for MP was 0.076g%. The same trend can be applied for hydrocortisone, in the viscous gel-like phase the solubility was 0.0238 g% *i.e.*% drug recovered >80% and in the micellar phase was 0.0075g%.

- *Mixtures of mono-, di-, and tri-glycerides and medium chain fatty acids*

Other experiments were performed using different mixtures of medium-chain fatty acids oil (Miglyol 812®) added to mixture of mono-, di- and tri-glycerides under the same conditions. At a ratio of 30:70/ M 812®: I 988®, the phases were identified as for the mixtures of mono-, di-, triglycerides only. At least 80% of the drug was in the viscous gel phase as shown in Figures 6.9-6.12. The viscous gel-like phase was not identified at a ratio of 30:70/ M 812®: I 988®. The volume of the viscous gel-like phase in 30:70 ratio of M 812®: I 988® would be higher than at the other ratio. Therefore, the concentration of the drug in the micellar and pelleted phase in 30:70 ratio of M 812®: I 988® would not be more than 15% each. Figures 6.9 and 6.10 showed that the concentration of BP in the micellar phase and in the precipitate was 0.021 g% and 0.065g% and testosterone was 0.0026 g% and 0.0075 g%. The concentration of hydrocortisone is 0.02599 g% and 0.0649 g% and MP in the micellar and pelleted phase is 0.071 g% and 0.023 g% as shown in Figures 6.11and 6.12 respectively. The concentration of BP in viscous gel-like phase of 70:30/ I988®: M812® ratio is 0.160g%, and for testosterone is 0.0401 g% respectively (Figures 6.9 and 6.10). The same observation was made for hydrocortisone 0.1504 g% and for MP 0.302 g%.

On the contrary, the ratio 70:30/ M812®:I988® resulted in three phases oily phase at the top followed by mixtures of interface and subphase and pellet phase at the bottom. The distribution of the drug between the phases was drug dependent. The drug was concentrated in the interface. The concentration of hydrocortisone was 0.0599 g% and 0.316 g% for MP. Testosterone and BP were more concentrated in subphase

0.0233 g% and 0.066g% respectively. On some occasions, it was difficult to extract the oily phase because of its small volume compared to the other phases.

Other ratios were tested 50:50/M 812®: I988®. The phases observed after ultracentrifugation were an oily phase at the top followed micellar phase, and a viscous gel-like phase. The volume of the subphase was >40% of the total volume and the viscous gel phase was <20%. Although the volume of the micellar pha: 204 higher than the viscous gely phase, the drug was highly concentrated in viscous gel phase. For example, the concentration of hydrocortisone in the micellar phase and viscous gel-like structure was 0.0287 g% and 0.215 g% respectively. The concentration of MP in interface and gel-like phase was 0.016 g% and 0.377 g% respectively. Similarly, for BP the solubility in interface and gel-like phase is 0.0287 g% and 0.215 g% respectively and for testosterone is 0.0021 g% and 0.0235 g% respectively (Figures. 6.9-6.12).

Another mixture was tested. The ratio of M 812®:I 988® was 62.5:37.5 (5:3), the same as in S-F formulations. Only two phases were observed; an interface phase and the rest was a clear micellar phase with some pellet. More than 80 % of the drug was concentrated in the micellar phase (subphase). Therefore, it can be concluded that for every 5 parts mixture of mono-, di- and tri-glycerides, 3 parts of medium chain fatty acid oils are needed to get the micellar solution (Figures. 6.9-6.12). For example, the concentration of BP was 0.229g% and for hydrocortisone was 0.0223 g%. The same trend observed for MP was 0.301 g% and for testosterone was 0.0365 g%.

- *SEDDS formulations*

The physico-chemical behaviour of the SEDDS formulation under standard pH-stat conditions was dependent on the contents of the formulations; therefore, the distribution of the drug would be expected to vary. As the hydrophilic content (surfactant, HLB >12 and co-solvent) increased, the micellar phase would be more

prevalent for example after the dispersion and digestion of Type III B formulations. Type III B contained at least 40% hydrophilic surfactant (HLB>12) and >40% hydrophilic cosolvent. After ultracentrifugation only a clear micellar phase was apparent with 100% of the drug dissolved in this phase as shown in Figures.6.13-6.16. For example, BP concentration was 0.459 g%, 0.0527 g% for testosterone, 0.0372 g% for hydrocortisone, and 0.534 g% for MP (Figures 6.13-6.16 respectively).

When type III A formulation was investigated where the concentration of the hydrophilic content was <45% (surfactant, HLB >12 and co-solvent), three phases can were apparent after ultracentrifugation. An interface phase at the top followed by a micellar phase and a pellet at the bottom. For almost all drugs, at least 50% of the drug dissolved in the subphase (micellar phase) and interface.

For example, the BP concentration was 0.213 g% in the micellar phase, hydrocortisone was 0.0193 g% and methyl paraben was 0.382 g% as shown in Figures 6.13, 6.15 and 6.16 respectively. Figure 6.14 showed that the concentration of testosterone was 0.0493g% in the interface. However in the case of type II where at least 40% medium chain fatty acids (M 812®) were mixed with hydrophilic surfactant (TTO®, HLB <12) <50%, four phases can be distinguished. At least 50% of the drug was dissolved in the micellar phase. For example, Figures 6.13 and 6.14 showed that the concentration of BP was 0.189 g% and 0.0296 g% for testosterone respectively. On the other hand, hydrocortisone and MP were more concentrated in the interface 0.0301g% and 0.292 g% respectively as shown in Figures 6.15-6.16.

S-F formulation produced three layers after ultracentrifugation, interface at the top, followed by subphase and precipitate. More than 50% of the drug was dissolved in the subphase and less than 20% in the interface. For example, the concentration of BP in micellar and precipitate was 0.240 g % and 0.132 g % respectively and for testosterone was 0.042 g % in micellar phase and 0.0018% in precipitate. The same

trend was observed for hydrocortisone 0.0194 g % and 0.0054 g % respectively. For MP the corresponding concentration was 0.396g% in the micellar phase.

6.4 Discussion

The above results indicate that the micellar hypothesis of fat absorption may not apply directly to all formulations. Its general principles are appropriate but the description which was presented 20 years ago was an oversimplification. The floating oil phase contained mostly unhydrolyzed TGs and DGs. The oil phase was a very small fraction of simulated intestinal contents with respect to volume in these experiments (Hofmann *et al*, 1964). Therefore, the concentration of the hydrophobic drugs was small compared with the other phases <10%. By chemical and phase analyses, Carey and his co-workers (1990) defined the floating phase (oil phase) that was comprised of oil-in-water emulsion particles with cores of TG, DG, and cholesteryl esters (CE) emulsified with a surface coat of partially ionised fatty acids (FA), MG, diacylphosphatidylcholine (PL) and bile salts. The turbid aqueous phase (micellar) contained mainly MGs and fatty acids with small amounts of TGs and DGs. The aqueous or 'micellar' phases can include at least two different aggregates: mixed disc-like micelles (multilamellar) saturated with lipolytic product and liposomes (unilamellar vesicles with hydrodynamic radii = 200–600 Å). Usually, in a static system, these different phases are in equilibrium with the micellar phase and are interconvertible.

In the intestinal contents, mixed lipids are formed by lipolysis at the same time as they are mixed with bile. The physical condition of lipids of bile is therefore of interest for the interactions. Concentrated bile, as it exists in the gall bladder is in the form of mixed bile salt-lecithin micelles in isotropic solution with particle size 40 – 60 Å (Arnsejö *et al*, 1969; Borgström, 1985). On dilution, the micellar bile salt concentration decreases with spontaneous growth in micellar size until the phase limit is reached and liposomal aggregates are formed. Hepatic bile exhibited similar behaviour which also can be predicted to form large mixed-disc micelles folding into

vesicles with particles of hydrodynamic radii of ≈ 350 Å (Arnsejö *et al*, 1969; Borgström, 1985). Hernell and co-workers showed by quasielastic light scattering (QLS) analysis of the components of subphases, that much larger proportions of lipids were solubilized by micelles than were dispersed as unilamellar vesicles. When followed as a function of time, vesicles frequently dissolved spontaneously into mixed micelles, indicating that, in the non-equilibrium *in vivo* conditions, the constituent's micellar phase was often not saturated with lipids. In an environment rich in of bile salts, unilamellar vesicles probably represent the primary dispersed product of human fat digestion and facilitate the dissolution of lipolytic products into unsaturated mixed micelles (Hernell *et al*, 1990). In nature, human intestinal contents are in a metastable state so that unilamellar vesicles of the subphases are likely to be appreciably smaller than the sizes predicted by 5–7 day equilibrium of the model systems. These vesicles often spontaneously dissolved into mixed micelles over time, strongly suggesting that they did not originate from supersaturated mixed micelles, in which case they would have been expected to grow in size (Staggers *et al*, 1990).

The turbidity and heterogeneity of the micellar phase seen here may have been due to low total lipid concentration in relation to the bile salt concentration (Borgström, 1985). Turbidity is known to occur when large liposomes are present and other aggregates form with a density close to that of the micellar solution. In the absence of sufficient bile salt and phospholipid, the micellar phase may contain liquid crystalline structures or other phases such as inversed L2 micelles containing fatty acids and MGs.

The present study shows the importance of MG, at low relative bile salt concentration, which may lead to the formation of liquid crystalline phases and optically isotropic L2 phase. The latter phase could be expected to be one of the major phases found in human intestinal content after administration of formulations rich in MGs. The presence of L2 is of interest because it is rich in hydrocarbon content and has properties resembling those of the oil phase. It may act to help

disperse the oil phase, increasing the velocity of lipolysis. In other studies performed for the present study, the solubility of DG and TG in bile salt solution was found, by contrast, to be extremely low. Excess DG and TG separated as an emulsion, but the emulsion contained undissolved DG or TG and did not contain bile salt or water. If moderate concentrations of DG and TG were present in intestinal content, these lipids should exist in the form typical emulsion droplets. Therefore, all the lipids present in intestinal contents are present either as emulsified droplets and the liquid phase between the droplet is ionic and non-micellar, or as an emulsified oil phase coexisting with a micellar phase (Hofmann *et al*, 1964). In the presence of calcium and insufficient amount of bile salts, the 'crusty' crystalline phase (most likely comprising insoluble Ca^{2+}-fatty acid soaps) may form pellets (precipitate). Pelleted lipids originated directly from the emulsifiers on the surface of the emulsion. Both unilamellar vesicle phase and a micellar subphase may be critically dependent upon a micellar bile salt concentration for both solubilization and multilamellar→unilamellar vesicle transformation (Staggers *et al,* 1990).

In some of the model systems studies here, an unexpected phase sedimented to the bottom of the tube as demonstrated in Figure 6.3. This was a viscous gel, believed to contain MGs and fatty acids. This unexpected phase separation is likely to have a considerable effect on the fate of drug dissolved in such formulations. Mixed mono- and di-glycerides are often good solvents for hydrophobic drugs and are used to increase the solvent capacity of lipid formulations. Our results suggest that use of high concentrations of MGs may be disadvantageous and could result in precipitation of drug.

Clearly, in view of the experimental findings described in this section, these results are compatible with the proposed model of the physical-chemical steps in fat digestion as shown in Figure 6.4 (Hernell *et al,* 1990; Embleton *et al*, 1997). In many adult humans, biliary lipids are mixed with pancreatic lipase/colipase complex, and together adsorb to the crude DG and TG surfaces, entering from the stomach. Lipase and colipase are secreted in equimolar proportions, giving duodenal concentrations of

$\sim 1–2 \times 10^{-7}$ M. They can release 150–300 μmoles of Sn–1 and Sn–3 long-chain fatty acids/min/ml of intestinal fluid (Carey *et al*, 1983). Biliary lipids, which may be a two-phase micelle-plus-vesicle system, stabilize the emulsion particles and reduce the emulsions sizes (Carey, 1983). The partially ionized FA normally enhances emulsification, these promoting binding of colipase-lipase complex to the emulsion surface and MG (Borgström, 1980). During lipolysis, lipids products, which may enhance the emulsification process, will locate mainly at the emulsion surface, as multilamellar liquid crystalline bilayers (Carey, 1988). As the lipolysis proceeds, the core of the emulsion droplet shrinks and parts of the surface coat pinch off as large liquid-crystalline structures (Hernell *et al*, 1990; Embleton *et al*, 1997). Postprandial gall bladder emptying causes a transient elevation in total bile acid concentration to 13 – 46 mM (Carey *et al*, 1983). Continuous production of unsaturated BS will catalyse the formation of small unilamellar vesicles from multilamellar liposomes and a two-phase system of vesicles and mixed micelles. Unilamellar vesicles (single-shelled liposomes) and micelles coexist in the aqueous-rich portion of duodenal contents and are readily dispersed in unsaturated BS into intestinal mixed micelles (Hernell *et al*, 1990; Embleton *et al*, 1997) (Figure 6.4). The structure of these large mixed micelles may be similar to, but larger than, that of the mixed disc model of BS-LC micelles (Carey *et al*, 1983). The intestinal absorption of FA, MG, Ch and other dietary and biliary lipids is probably in the form of micelles due to their large number and unexpectedly small size thereby giving more rapid diffusive access to the mucosal surface. However, absorption could take place from unilamellar and, perhaps, even multilamellar vesicles (Hernell *et al*, 1990). Therefore, saturation of mixed micelles by unilamellar vesicles produces the most favourable thermodynamic condition for maximizing lipid absorption rates from the upper small intestine; further; lipolytic products dispersed as uni-and multilamellar vesicles may explain the slow, but efficient, fat absorption that takes place from the entire small intestine in BS-deficiency cases. Lipid digestion products pass across the intestinal unstirred water layer. For LCT and cholesterol, passage across the unstirred water layer is rate limiting, whereas the brush border membrane limits passage of short-and medium

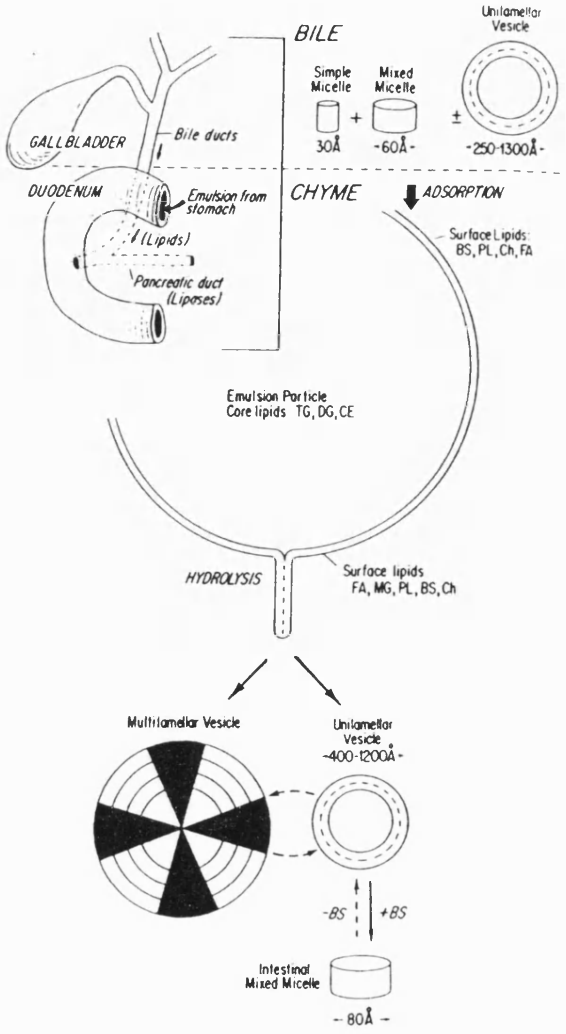


Figure 6.4 Composite and simplified model of the physicochemical changes in duodenal content digestion. Postprandial duodenal contents exposed to lipase-colipase adsorb to the surface of crude emulsion particles entering the duodenum from the stomach. Lipolytic products in great excess on the emulsion surface lead to shrinking of the core of the oil and building up of FA, PL, and MG with enormous surface pressure. Surface pressures cause dissociation of multilayers of lamellae from the surface, which bud off as unilamellar vesicles. Subsequently they are solubilized by bile to form mixed micelles (Carey, 1983; Hernell *et al*, 1990, and Embleton *et al*, 1997).

chain fatty acids. Within the unstirred layer, an acidic microclimate aids micellar dissociation so that protonated, and to a lesser extent, non-protonated monomers then pass across the intestinal brush-border membrane (Thomson *et al*, 1993).

6.5 Conclusion

The previous observations provide a fundamental framework for understanding the physical-chemical state of the fate of lipid excipients and/or SEDDS formulations in standard pH-stat solution under standard pH-stat conditions. The data obtained here has been correlated to the study on the upper intestinal contents reported by Staggers *et al* (1990) and agrees with their findings. However, a new viscous gel-like phase was identified; believed to comprise monoglycerides. This unexpected phase separation may have a considerable effect on the fate of drug dissolved in some formulations. The ability of lipid digestion products to keep the drug in solution is highly dependent on the physico-chemical properties of lipid themselves. Bile acid solubilization results in mixed micelles and liposomes, gel and liquid crystal phases.

Type III B can be a good solvent for both steroids and hydroxy benzoate derivatives after lipolysis because of the production of micellar phase (subphase). Therefore, the drug will be ready for absorption. The other SEDDS formulations they produced a precipitate after lipolysis which may entrapped a considerable amount as in case of hydroxy benzoate derivatives. The presence of bile salts was necessary in case of type I, II and S-F formulation to enable the formation of mixed micellar phase.

MCT lipolytic products are mainly subphase and precipitate. The fate of hydrophobic drugs in MCT could be absorption through the mixed micellar phase rather than precipitation as in case of hydroxy benzoate derivatives. However, in case of steroids the fate of the drug is uncertain because of their low solubility. The presence of gel-like phase after the lipolysis of mixed mono-, di-, and tri-glycerides entrapped a significant amount of the hydrophobic drugs. The fate of the drug in gel-like phase can be precipitation rather than absorption because it is a swollen MG enriched phase.

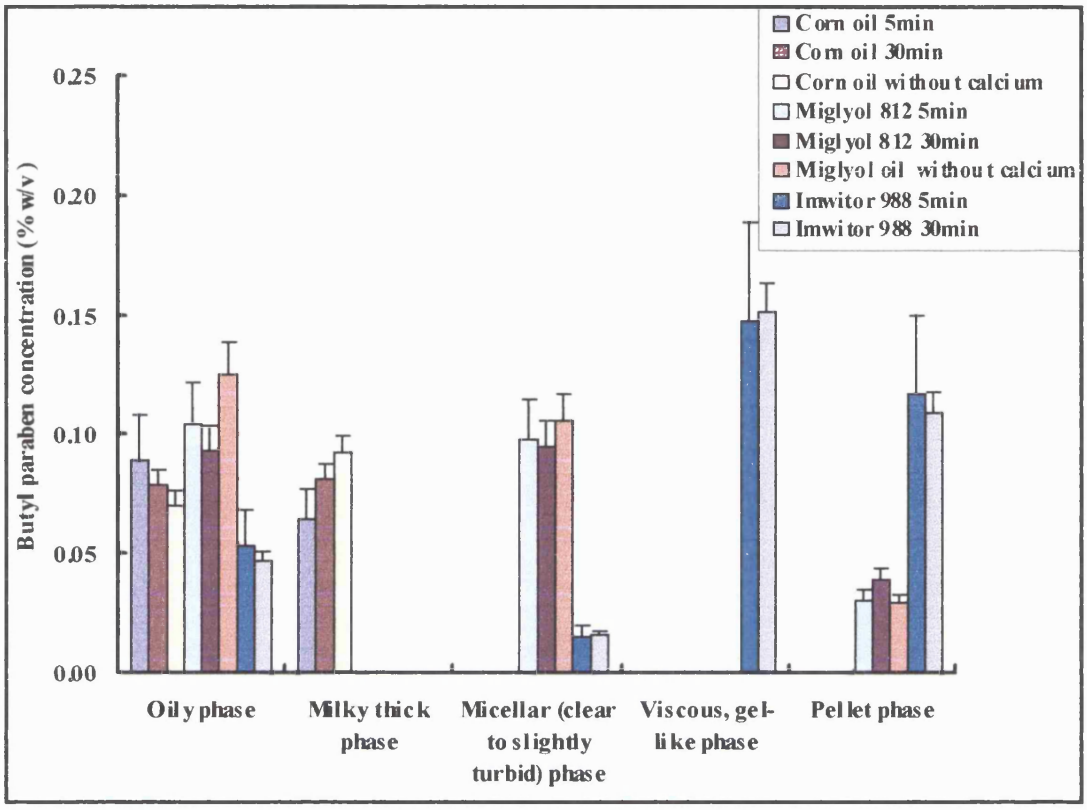


Figure 6.5 Determination the fate of butyl paraben in lipid excipients (LCT{Corn oil}, MCT {Miglyol 812®}, and mixture of mono-, di-, and tri-glycerides {Imwitor 988®}) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15 mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g). LCT (Corn oil) and MCT (Miglyol 812®) were repeated under the same standard pH-stat conditions in the absence of calcium (the error bars represent the standard deviation of three experiments).

- **5** min = samples were ultracentrifuged at 104630 g for 5 min at 37°C.
- **30** min = samples were ultracentrifuged at 104630 g for 30 min at 37°C.

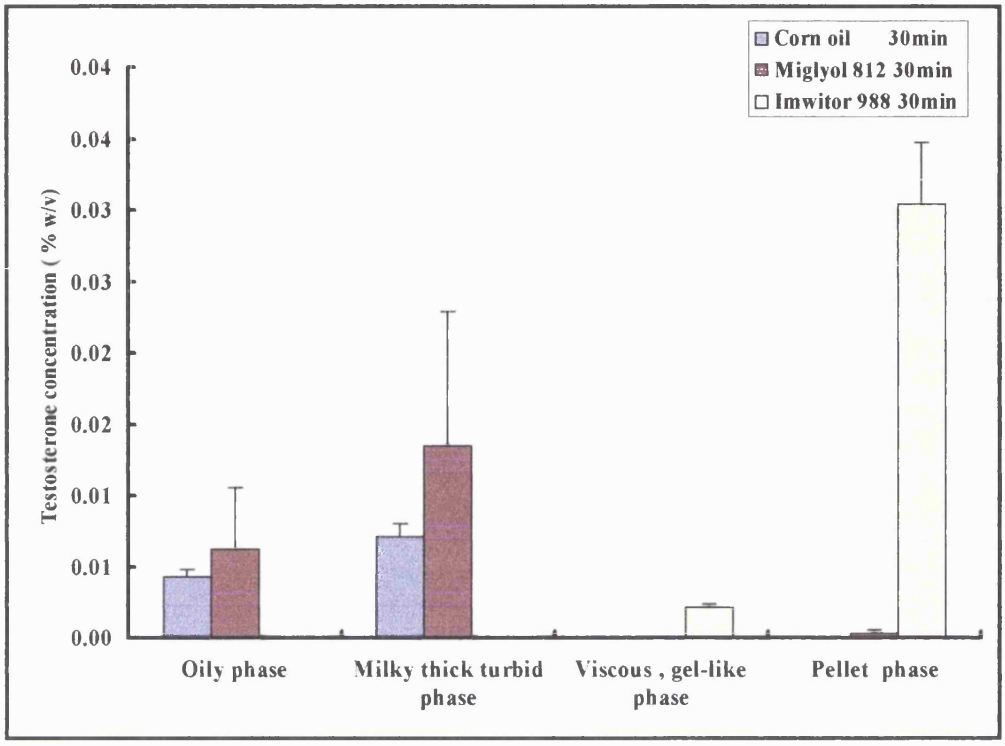


Figure 6.6 Determination the fate of testosterone in lipid excipients (LCT{Corn oil}, MCT {Miglyol 812®}, and mixture of mono-, di, and tri-glycerides {Imwitor 988®}) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15 mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) for 30 min (the error bars represent the standard deviation of three experiments).

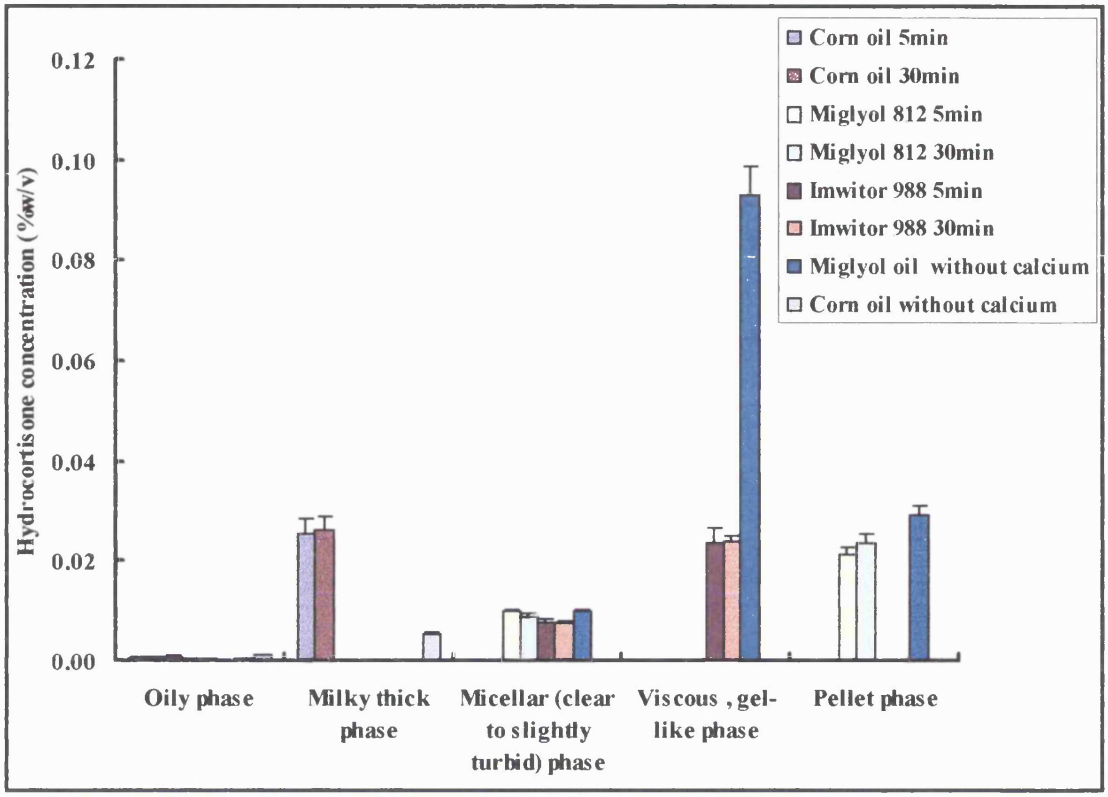


Figure 6.7 Determination the fate of hydrocortisone in lipid excipients (LCT{Corn oil}, MCT {Miglyol 812®}, and mixture of mono-, di-, and tri-glycerides {Imwitor 988®}) phases after digestion for 30 min under standard pH-stat conditions (4g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) (the error bars represents the standard deviation of three experiments).

- **5** min = samples were ultracentrifuged at 104630 g for 5 min at 37°C.
- **30** min = samples were ultracentrifuged at 104630 g for 30 min at 37°C.

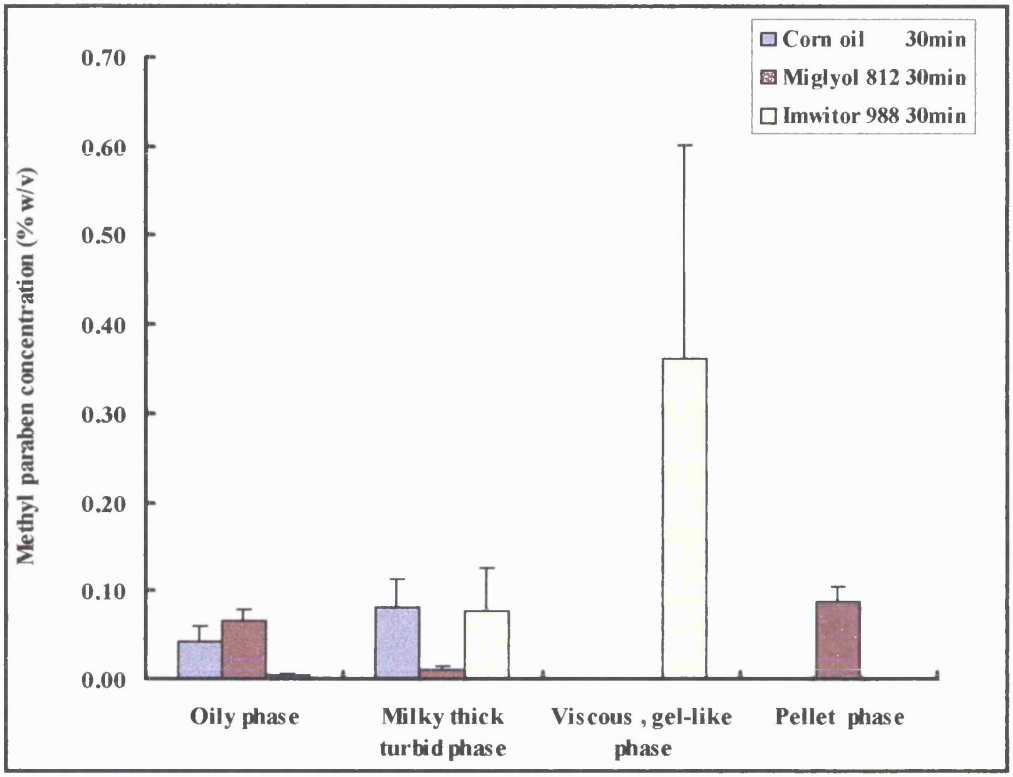


Figure 6.8 Determination the fate of methyl paraben in lipid excipients (LCT{Corn oil}, MCT {Miglyol 812®}, and mixture of mono-, di, and tri-glycerides {Imwitor 988®}) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15 mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) for 30 min (the error bars represent the standard deviation of three experiments).

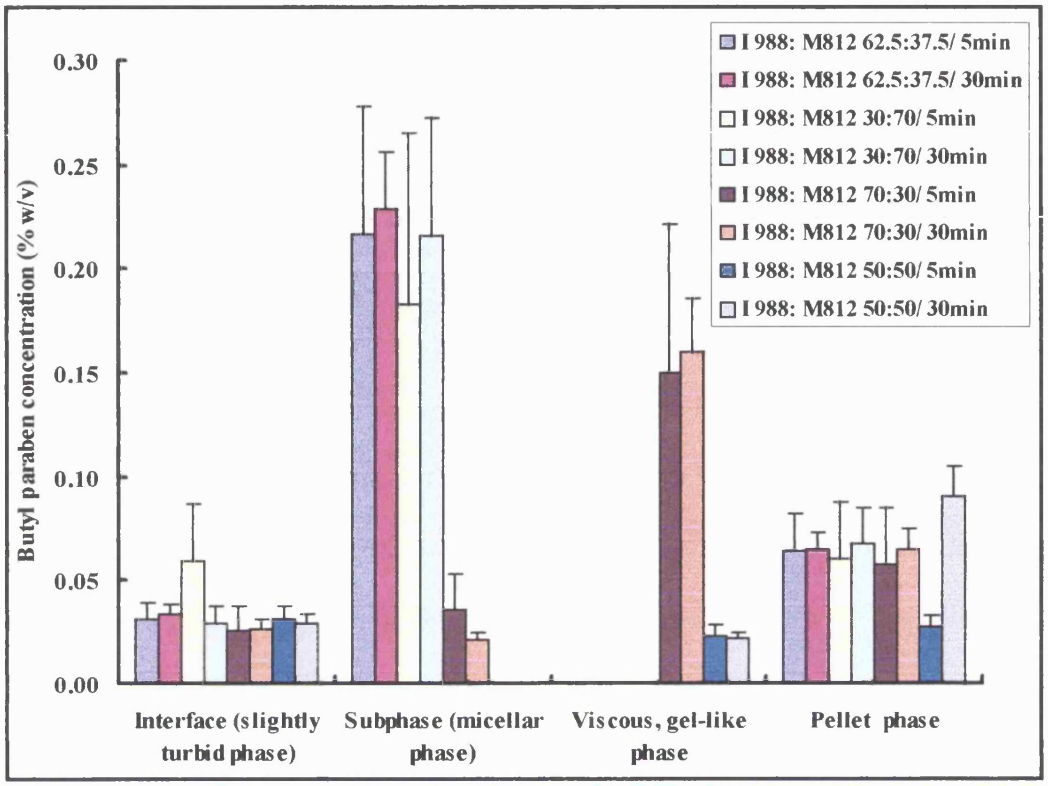


Figure 6.9 Determination the fate of butyl paraben in mixtures of MCT (Miglyol 812) and mixtures of mono-, di-, and tri-glycerides (Imwitor 988®) (table 6.1) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) (the error bars represent the standard deviation of three experiments).

- **5** min = samples were ultracentrifuged at 104630 g for 5 min at 37°C.
- **30** min = samples were ultracentrifuged at 104630 g for 30 min at 37°C.

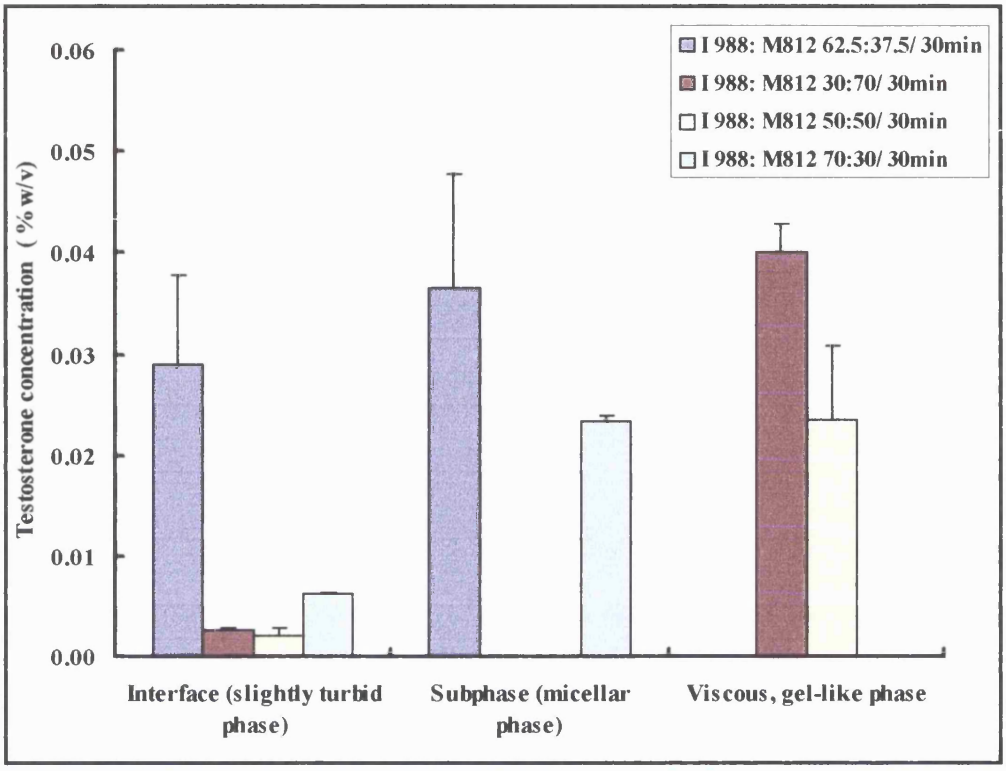


Figure 6.10 Determination the fate of testosterone in mixtures of MCT (Miglyol 812®) and mixtures of mono-, di-, and tri-glycerides (Imwitor 988®) (table 6.1) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) for 30 min (the error bars represent the standard deviation of three experiments.

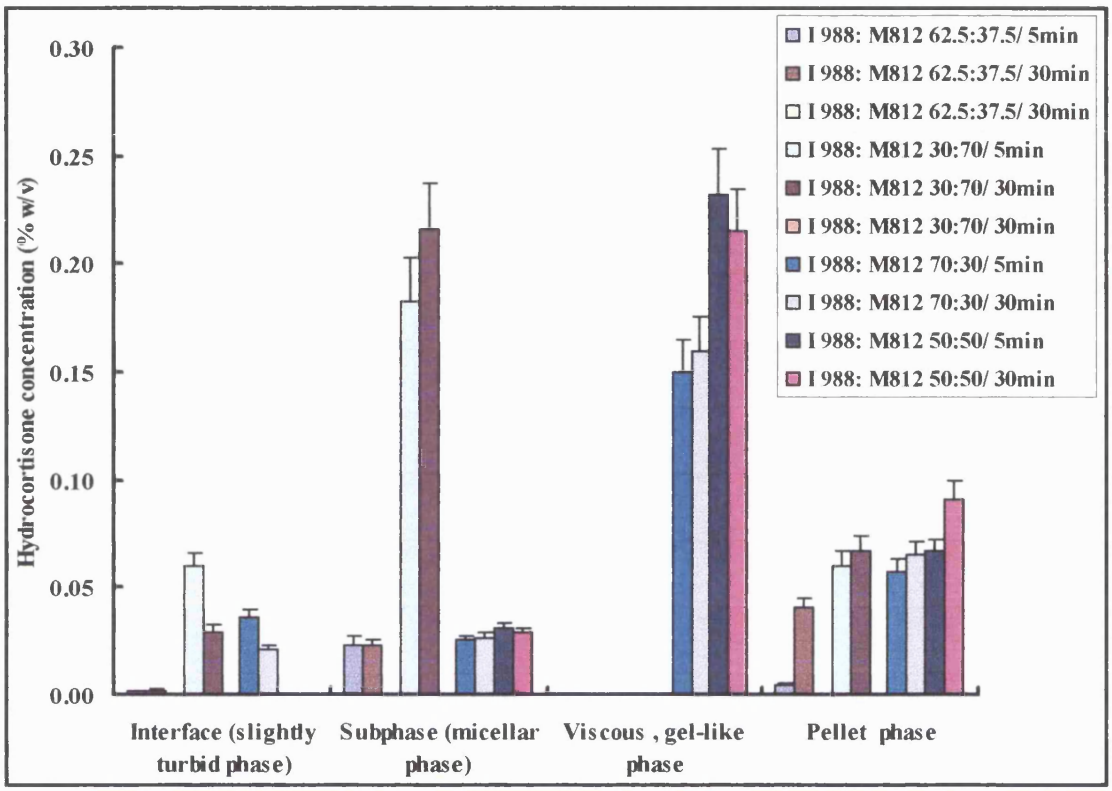


Figure 6.11 Determination the fate of hydrocortisone in mixtures of MCT (Miglyol 812®) and mixtures of mono-, di-, and tri-glycerides (Imwitor 988®) (table 6.1) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) (the error bars represent the standard deviation of three experiments).

- **5** min = samples were ultracentrifuged at 104630 g for 5 min at 37°C.
- **30** min = samples were ultracentrifuged at 104630 g for 30 min at 37°C.

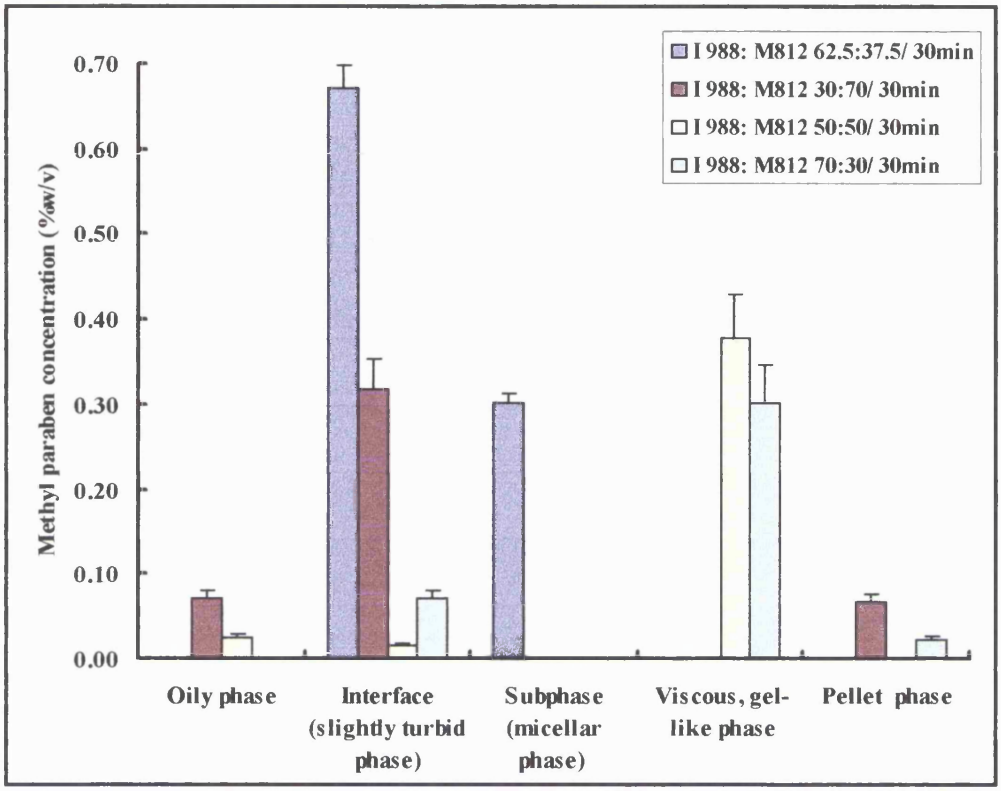


Figure 6.12 Determination the fate of methyl paraben in mixtures of MCT (Miglyol 812®) and mixtures of mono-, di-, and tri-glycerides (Imwitor 988®) (table 6.1) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) for 30 min (the error bars represent the standard deviation of three experiments).

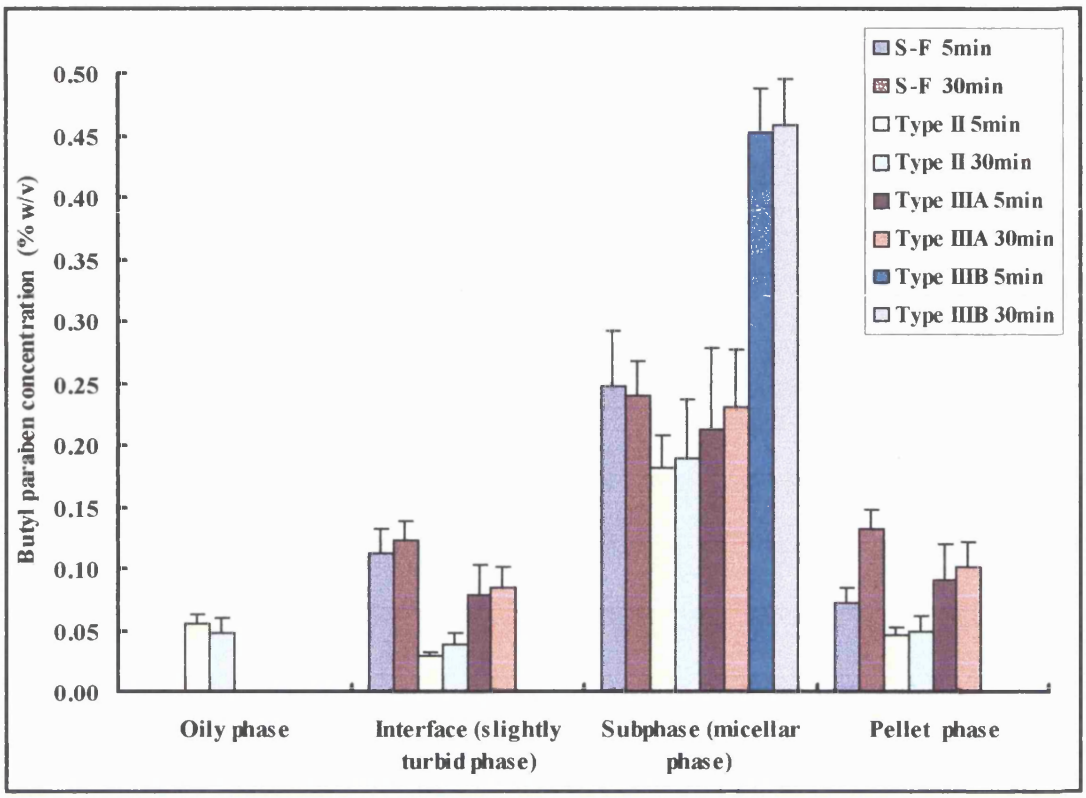


Figure 6.13 Determination the fate of butyl paraben in SEDDS formulations (table 6.1) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) (the error bars represent the standard deviation of three experiments).

- **5** min = samples were ultracentrifuged at 104630 g for 5 min at 37°C.
- **30** min = samples were ultracentrifuged at 104630 g for 30 min at 37°C.

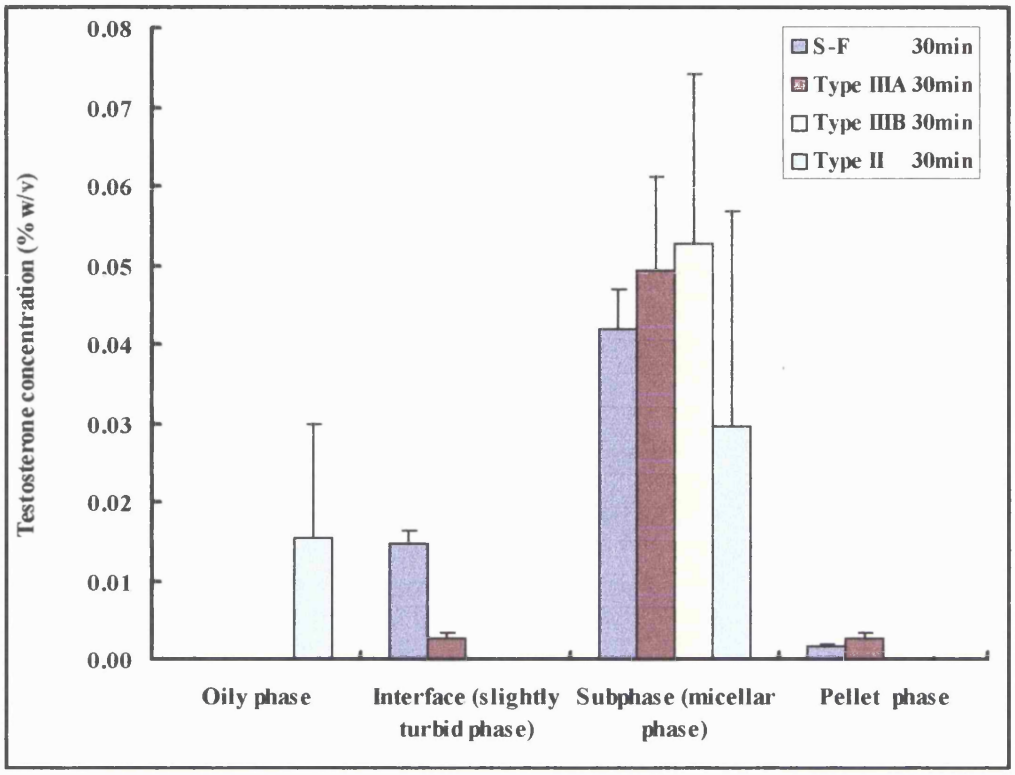


Figure 6.14 Determination the fate of testosterone in lipid excipients in SEDDS formulations (table 6.1) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) for 30 min (the error bars represent the standard deviation of three experiments).

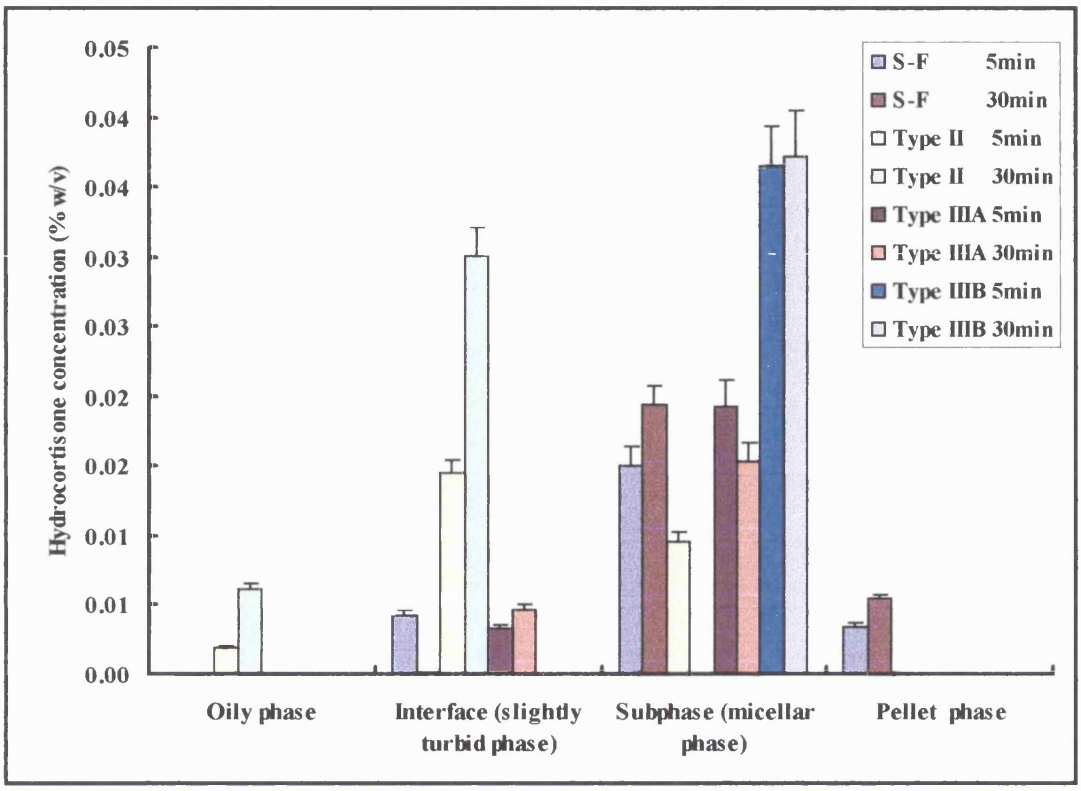


Figure 6.15 Determination the fate of hydrocortisone in SEDDS formulations (table 6.1) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) (the error bars represents the standard deviation of three experiments).

- **5** min = samples were ultracentrifuged at 104630 g for 5 min at 37°C.
- **30** min = samples were ultracentrifuged at 104630 g for 30 min at 37°C.

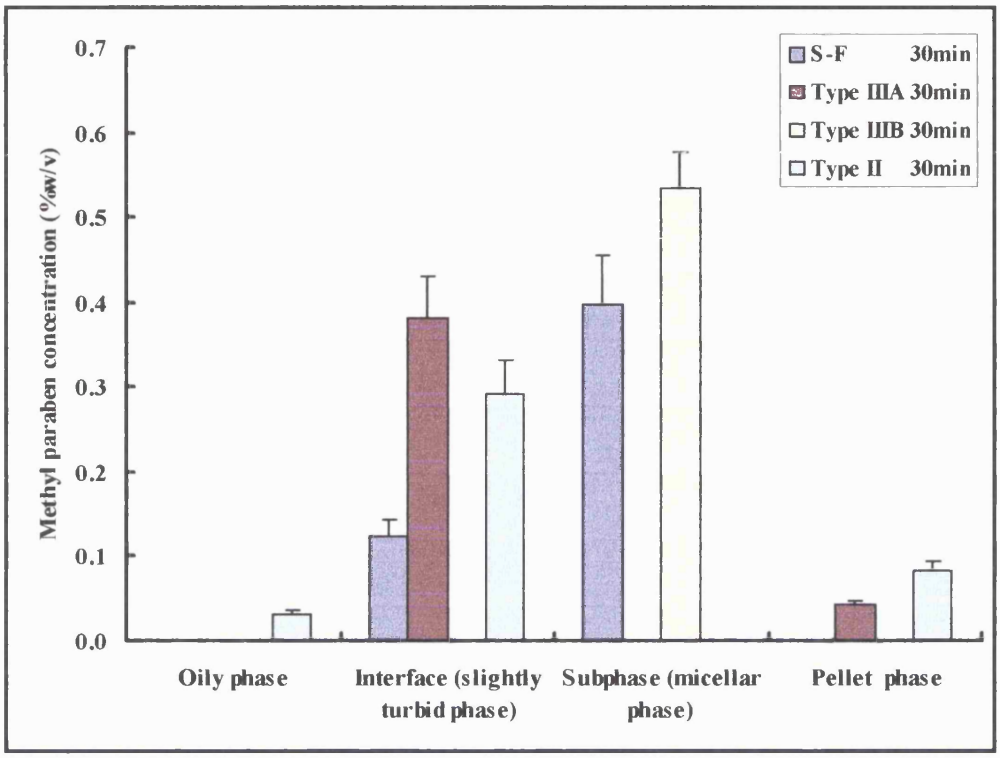


Figure 6.16 Determination the fate of methyl paraben in SEDDS formulations (table 6.1) phases after digestion for 30 min under standard pH-stat conditions (4 g of lipid excipients in the presence of 6 TBU / mg pancreatin enzyme in tris-maleate buffer (pH 7.0) in the presence of 15mM NaTDC and 3.75 mM PL at 37°C). Samples were analysed using HPLC method after ultracentrifugation (104630 g) for 30 min (the error bars represent the standard deviation of three experiments).

Chapter 7

General discussion and conclusions

The work performed in this thesis has provided new information regarding the use of lipid-based formulations for drugs which exhibit dissolution rate-limited absorption. In general, hydrophobic drugs with aqueous solubilities lower than 100 μg / ml often present dissolution limitations to absorption. The dose: solubility ratio of the drug provides an estimate of the volume of fluids required to dissolve an individual dose, and when this volume exceeds 11 litres, dissolution is often problematic (Dressman *et al*, 1986). When such a formulation is released into the lumen of the gut it disperses to form a fine emulsion, so that the drug remains in solution in the gut, avoiding the dissolution step which frequently limits the rate of absorption of hydrophobic drugs from the crystalline state. Generally, this can lead to improved bioavailability, and/or a more consistent temporal profile of absorption from the gut.

Steroids derivatives were used as models of hydrophobic drugs with log *P* between 1-4 and compared later with hydroxy benzoate derivatives in some studies. Hydroxy benzoate derivatives were used because of their low molecular weight and the expectation that they might exhibit better solubility than steroids in lipid excipients and formulations. The choice of model drugs for academic studies presents practical problems, and neither steroids or benzoates are ideal models. Neither is a good representation of the new chemical entities (NCEs) which are emerging as drug candidates in 2002. Choice of model drug was limited by cost and availability. A series of hydrophobic heterocyclic compounds with weakly basic amine groups would be better models of typical NCEs.

SEDDS formulations, consisting of emulsion pre-concentrates which produce fine oil- in- water or microemulsion when introduced an aqueous phase, are suitable for encapsulation thereby providing precise and convenient unit dose forms. However, constraints on solubility, potential interaction with excipients and physical stability

limitations all impact on the utility of such formulation approach. Recently, three main criteria with which lipid formulations can be distinguished were described by Pouton (1999) to aid interpretation of comparative bioavailability (discussed earlier in chapter 1). Through my research, I have tried to verify these criteria's and added to the classification with a new class of formulation described here as a Surfactant-Free (S-F) system.

Self-emulsifying formulations of new lipophilic benzodiazepine compounds which can be filled into hard or soft gelatin capsules for oral administration have been recently patented (Lievens *et al*, 1992). These formulations contain propylene glycol, polyglycolyzed glycerides, such as Labrafil M 2125 CS or M 1944 CS or Labrasol in combination with Tween 80 and are claimed to be useful for the treatment of pain and panic or anxiety (Constantinides, 1995). Improved dissolution and oral absorption of indomethacin in the rat from a self-microemulsifying drug delivery system incorporating polyglycolyzed glycerides as compared to an aqueous suspension of the drug has also been demonstrated (Farah *et al*, 1994).

Surfactants are known to increase the permeability by disrupting the cell membrane (Swenson *et al*, 1992). The main rate-limiting barrier for the drug absorption/ diffusion is the single layer of intestinal epithelial cells that covers the luminal surface of the intestinal wall. In addition, the pre-epithelial, unstirred aqueous layer presents a barrier hinder the poorly soluble drugs from reaching the absorption site (Arthursson *et al*, 1991). For the majority of drugs, absorption occurs *via* passive transcellular transport and the paracellular transport is limited due to tight junctions between the cells. Lindmark *et al* (1995) have used the medium chain fatty acids (*e.g.* sodium caprate) to enhance the absorption of hydrophilic drugs by modifying the tight junctions. It was reported that intestinal mucosa is frequently subjected to dietary induced damage (*i.e.* by bile salts, fatty acids and monoglycerides) and that mechanisms have evolved for rapid repair (Humberstone *et al*, 1997). Surfactants monomers are capable of partitioning into clear membrane where they can form polar defects in lipid bilayer. At high surfactant concentration in the cell membrane,

surfactant-surfactant occurs, and the membrane can be dissolved into surfactant-membrane mined micelle (Swenson *et al*, 1992).

With an increasing number of lipid excipients available for use, there is a need for more information to allow optimal choice of excipients. More knowledge is needed regarding surfactants in particular, of their toxicity and how important they are to the performance of the formulation. Due to these concerns of using surfactant for chronic administration, one motivation for the formulator is to find an alternative formulation using lipid excipients which have GRAS (generally regarded as a safe) status.

It is clear from the phase diagrams that the S-F formulations are clear, transparent, and thermodynamically stable. They are typically isotropic mixtures of medium chain oils < 40% (Miglyol 812®), mixture of mono-, di-, and tri-glycerides > 40% (Imwitor 988®) and water soluble co-solvent < 30 % (Transcutol P®, PG, PEG 400). As can be seen, the area of mutual solubility produced by the S-F systems is extended over a wide range of compositions controlled by both the polarity of the cosolvent and the mono-, di-, and tri-glycerides content of the oil.

The processes by which lipid solubilized hydrophobic drug molecule eventually finds its way from its original bulk oil environment to the intestinal membrane, where it then diffuses across the mucosal bilayer to ultimately appear in the blood or the lymph, are extremely complex and likely to be critical components in determining bioavailability. Compared to these events, the chemical factors controlling drug solubility in a homogeneous lipid solvent seem an almost inconsequentially minute piece of the puzzle (Anderson, 1999). The essential first steps in a lipid-based strategy are to dissolve the drug in lipid excipients and/or SEDDS formulations (intrinsic solubility) in order to determine the solvent capacity of the formulation, and to determine whether the drug remains in solution on dispersion in aqueous solution.

SEDDSs formulation and the individual excipients are viscous formulations; therefore, it is not easy to predict the length of time required reaching equilibrium. It was convenient to use gentle heating to speed up the dissolution process and it was important to make sure that the solubility measurements after re-equilibration at room temperature were true values of solubility rather than supersaturated systems.

Therefore, the protocol used for determining a single point of intrinsic solubility was to mix lipid excipients at elevated temperature in the presence of the drug for 2–3 minute then left for equilibration for at least 48-96 hours before further analysis was undertaken.

A recent study reported by Nakervis *et al* (1996) used retinoids (isotretinoin log $P \approx$ 6.8, etretinate log $P \approx 7.8$ and temarotene log $P \approx 8.7$) as highly lipophilic molecules that are known to be transported in the intestinal lymph oral administration. Lipid solubility showed a general increase with increasing log P of the retinoids. The most lipophilic retinoid, temarotene, showed solubilites 109.5 mg/ml (linoleic acid) and 170.6 mg/ml (Miglyol 812®), whereas the least lipophilic retinoid, isotretinoin showed much lower solubility between 5.1 (cottonseed oil) and 30.5 mg/ml (linoleic acid). Lymphatic uptake of temarotene was 4000-times and from etratinate 1000-times greater than isotretinoin. The rank order of increasing lymphatic uptake from each of the three oils shows an inverse relationship with solubility of the retinoid in each of the oils.

For lipid formulation it is necessary to understand how the intrinsic solubility will be affected by mixing various components, such as oils, cosolvents and surfactants, how solubility is related to choice of surfactants, and how solubility is related to molecular weight, melting point and log P of the drug. The ability to make predications from solubility studies in single excipients would allow the formulator to make an early choice between the types of lipid formulations.

The results revealed that the mixed glyceride excipients, Imwitor 988® and Capmul MCM®, were particularly good solvents for all of the cotricosteroids. There was a

marked difference in solubility of steroids in MCT oil, and consequently it was considered that co-solvent might improve the solvent capacity of triglyceride-based systems. Surfactant-free formulation are typically composed of mixtures of Transcutol P® < 30 %, Imwitor 988® >50 % and Miglyol 812® >30 %. Surfactant-free (type IV) formulations were generally better solvents than SEDDS (type II) formulations but were generally more or less superior solvents to mixed glycerides of mono, di and tri-glycerides (Imwitor 988 ®) and (Capmul MCM®) alone. Type III SEDDS, which are hydrophilic, were also better solvents for most of the steroidal compounds than type II SEDDS. Although the homologous series of steroids span a considerable range of log *P*, their solubilities across a range of excipients and formulations were surprisingly similar, generally ranging from 0.2-2.1 g % w/v. Hydrocortisone acetate had the lowest solubility in most systems than the other steroids, and generally, progesterone had the highest solubility. Contrary to the observation with hydroxy benzoate derivatives, as Log *P* increased the solubility decreased in most systems.

Recently (Kommuru *et al*, 2001) reported a two fold increase in the bioavailability of CoQ_{10} (known as ubidecarenone used for treatment of cardiovascular disorder and as antioxidant) in SEDDS system compared to a powder formulation. They showed that the solubilities of CoQ_{10} in medium chain fatty acid glyceride and Myvacet® 9-45 (diacetylated monoglycerides of C_{18} fatty acids) were higher than solubilities in long chain fatty acid glycerides.

The solubilities of hydrophobic drugs in aqueous dispersions of SEDDS formulations are poorly understood and documented. For hydrophobic drugs, the best strategy is to keep the drug in solution during its passage through the gut, to avoid the dissolution step. As well as presenting the drug in solution, the formulations would ideally be finely dispersed within the gut to ensure that drug can be made available for absorption from the lumen of the gut, partitioning from the reservoir of dissolved drug. Type I or type II lipid formulations are good candidates to avoid precipitation of hydrophobic drugs. However, these systems have limited solvent capacity for

drugs such as cyclosporin A and steroids with high log *P*, which has encouraged the use of type III systems (Pouton, 1999). Type III systems upon dilution will form supersaturated solutions, but this need not necessarily lead to a rapid precipitation. The key issue is that if the drug precipitates on dilution of the formulation in the gut, then any advantage is likely to be lost.

The fate of each type of SEDDS formulations in the presence of the steroids derivatives upon dilution in water and/or tris-maleate buffer (pH 6.5) was determined. Self-emulsifying systems are expected to disperse rapidly within the contents of the stomach, which indicates that the formulation will empty into the intestine in a manner similar to the emptying of aqueous solutions. Consequently, the rate of absorption from a type II and a type III system is likely to be rapid, particularly when administered to a fasted stomach. Hypnotics and analgesics are examples of drugs which may benefit from these formulations because they require rapid onset of action. On the contrary, these formulations may be disadvantageous for drugs with low therapeutic indexes. Type I formulations are likely to be dependent on digestion by lipolysis, is a process which takes place in the small intestine. The drug will be solubilized in mixed micelles of bile salts as long as the drug does not precipitate during digestion. Therefore, the drug will not have an unusually rapid onset of action. A major factor is the digestibility of the formulation. If the formulation is non-digestible then a colloidal state must be provided by self-emulsification, whereas if digestible oils are used, the colloidal state can be obtained by natural digestion. The overall bioavailability can then be equivalent to that achieved by type II or type III formulation (Pouton, 1999).

Steroids are non-electrolytes; their behavior in the small intestine can be compared with weak bases with a pK_a less than 7. Weak bases will not be highly soluble in the small intestine; they may dissolve in the stomach but may precipitate after gastric emptying. Therefore, weak bases may benefit from reformulation into lipid systems, which can give a reservoir of drug dissolved in either lipid or micellar solution.

The key issue in relation to the use of type III A or B systems is the hydrophilic content (surfactant and co-solvent) that can keep the drug in solution. In practice, the presence of co-solvent and surfactant can improve the solvent capacity, but the instant they are diluted they can migrate to the aqueous phase solubilize leaving large amounts of the drug to precipitate. This effect is expected to be more pronounced in the presence of co-solvent, whereas a surfactant may be capable of forming a micellar solution. The results suggest that even in the presence of surfactant there can be a significant loss of the solvent capacity leading to precipitation of drug.

At one extreme, when a hydrophobic drug is formulated in an aqueous co-solvent (such as propylene glycol or PEG 400), there is usually a drastic loss of solvent capacity and consequent precipitation of drug. The amount of the drug which stays in aqueous solution of hydrophilic cosolvents can be estimated from the solubilization capacity (σ). The solubilization capacity is relatively log *P* dependent. As log *P* increased the solubilization capacity increased and the increased was significantly noticed upon dilution in tris- maleate buffer (pH 6.5). Hydrocortisone acetate was the least affected by a co-solvent system which could be explained by its molecular structure. At the other extreme (lipophilic), if a drug is formulated in a triglyceride (TG) oil there will be no dissolution of TG, and no precipitation of drug because the MCT oil will still be present as a separate phase, though possibly in a partially emulsified state.

It can be anticipated that self-emulsifying formulations may also lead to a loss of solvent capacity, depending on the extent to which water-soluble components are used. Thus, type II SEDDS and S-F formulation should be less susceptible to precipitation than type III SEDDS. The inclusion of hydrophilic surfactants and co-solvents could well lead to precipitation depending on the proportion of water–soluble components used. If the formulation contains a high proportion (Type III B) then precipitation is to be expected. Whether this becomes a problem will depend on the rate of crystallization in the gut.

The amount of drug recovered after dispersion of steroids in aqueous solution of CRH 40® or T80® was more than expected due to the micellization effect. The micellization behaviour can be explained by the favourable entropy changes (Barry *et al*, 1976). Two opposing factors are involved. Insertion of solubilized molecules in micelles restricts molecular movement, *i.e.* a more ordered state, and provides a negative change in entropy. An opposing effect is that as relatively non-polar molecules leave the aqueous phase for the micelle, the configurational entropy of the water molecules increases due to the break up of iceberg structure surrounding non-polar groups, *i.e.* a less ordered state produces a positive change in entropy.

For lipophilic steroids, the crowding effect predominates and hence a negative change in entropy occurs on solubilization. It is likely that more lipophilic steroids do not entirely lose their water structuring on entering the micelle because the region into which they are most likely to be incorporated, *i.e.* the outer layer of the polyoxyethylene shell, is heavily hydrated by water molecules physically trapped between the chains. Therefore, there would be little change in entropy, due to loss of water structure, but a decrease in entropy due to restriction of the solubilized molecules in the micelle. Relatively high log P (> 3) steroids, testosterone and progesterone, are solubilized in the less hydrated region of the polyoxyethylene shell, close to the hydrocarbon core, which is largely purely polyoxyethylene. When these steroids leave the aqueous phase, water structure breaks down and is not reformed in the micelle because there is little water at this site. Therefore solubilization of non-polar steroids increases entropy, due to break up water structure, which is greater than loss in entropy due to restriction of steroids within the micelle an hence a net positive change in entropy occurs (Barry *et al*, 1976; Anderson, 1999).

The first estimation to make from the equilibrium solubilities is what happens in the expected worst case represented by formulation as a simple co-solvent solution. Since the steroids are only soluble at approximately 1.1% w/w in each formulation, then the maximum dose, which can be administered in solution, is approximately 10mg/ml, *i.e.* 11mg in a 1ml capsule. This means that a formulation of 1g diluted to

100ml must be able to dissolve the drug at 11mg/100ml (0.011% w/w; 0.11mg/ml). As a result 1 g of S-F formulations composed of 30 %< of mixed glycerides mono-, di-, and tri- glycerides, < 30 % medium chain oil with or without < 30 % hydrophilic surfactant could keep the drug into solution (20mg/ 100ml) because steroids are soluble at approximately 2.0% w/v in S-F formulations.

The presence of bile may improve the bioavailability of poorly water soluble drugs by enhancing the rate of dissolution and/or solubility. An increase in the rate of dissolution can occur via (i) a decrease in the interfacial energy barrier between solid drug and the dissolution medium (*via* enhanced wetting), leading to an effective increase in surface area, or (ii) an increase in solubility *via* micellar solubilization within the bile salt micelle *i.e* a decrease in the apparent diffusion coefficient of the drug. The changes in the dissolution rate of poorly soluble drugs due to wetting or solubilization is compound dependent due to the specificity of the interactions associated with these processes (Bakatselou *et al*, 1991).

In the small intestine, amphiphilic bile components such as bile salts, lecithin, lipid digestion products such as fatty acids and monooleins can enhance drug solubility. When these substances are present in concentration higher than their critical micellar concentration (CMC), micellar solubilization of the drug can occur (Aungst, 1993; Charman *et al*, 1997; Humberstone *et al*, 1997; Hörter *et al*, 2001). Solubilization into simple bile salt micelles has been reported for many poorly soluble drugs including griseofulvin, glutethimide, digoxin, leucotriene-D antagonists and gemfibrozil (reviewed by Hörter *et al*, 2001). The addition of physiological bile salt to aqueous media increased drug solubility up to 100- fold.

The addition of lecithin, monooleins, long chain fatty acids and/or triglycerides, in several cases, to bile salt solutions produced further enhanced in drug solubility. Addition of lecithin causes an increase in the molecular weight of micelles from 6000 to 150000 Da (Shankland, 1970). In most cases, there is correlation between size and solubilization capacity of mixed micelles, but this dependent on the mechanism by

which the drug is solubilized. The ratio of bile salt to lecithin may also influence the extent of solubilization. Rosoff *et al*, for example, showed that the greater the molar ratio of lecithin to bile salt, the higher the solubility of diazepam (Rosoff *et al*, 1980).

The effects of formulating drugs in SEDD formulations are far from predictable, although strong evidence exists in the literature of the potential usefulness of the approach. Charman *et al* (1992) examined the lipophilic antiviral compound WIN-54954 administered in a formulation containing a medium chain triglyceride and a nonionic surfactant. The SEDDS was compared to a PEG-600 formulation. No significant difference in mean bioavailability was seen between the two systems but greater reproducibility was noted for the SEDDS. In contrast, Shah *et al* (1994) examined the bioavailability of Ro-150778, a highly lipophilic naphthalene derivative, demonstrating a fourfold greater bioavailability for the SEDDS formulation than a PEG- 400 solution and a 20-fold greater bioavailability than from standard tablet. Gershanik and Benita (1998) have compared progesterone absorption in female rats from a range of formulations, including SEDDS, which form positively and negatively charged droplets. The positively charged systems, which include the cationic lipid oleylamine, showed the greatest bioavailability with a solution in PEG-300, although whether this reflection of the surface charge or surface composition is not clear (Craig *et al*, 2001).

As physiological lipid processing has been suggested to enhance bioavailability from a lipid-based formulation, the possible rate of digestion of lipid-based formulations in the duodenum was assessed in this study using an *in vitro* model of lipolysis. This model enabled examination of the rate at which a lipid substrate was digested by lipase, in the presence of non-ionic surfactants likely to be included within a lipid-based formulation.

An evaluation method of the hydrolysis of lipid formulations containing glycerides, for administration to the gastrointestinal tract, in the presence of pancreatic lipase, was established. A general method, which uses a pH-stat to determine liberation of

free fatty acid, has been available for some time (MacGregor *et al*, 1997) but, given the complexity of the gut lumen, there is still much to learn about the use of *in vitro* models of lipolysis. This study aimed to investigate the variables which can affect lipolysis, and the significance of these variables on hydrolysis of long-chain triglycerides (LCT), medium-chain triglycerides (MCT), tributyrin (SCT) and mixed mono- and di-glycerides (MG). The activity of pancreatic lipase was expressed in terms of *Tributyrin Units or TBUs.* One TBU is the amount of enzyme that can liberate 1μmole of fatty acid per minute from tributyrin at 25 °C and pH 8.5 in the presence of 5mM CaCL2.2H 2O, 150mM NaCl. Normal human duodenal contents are reported to contain 1000 TBUs of colipase-saturated lipase per ml (Borgström *et al*, 1984). The optimum pH for lipase activity will depend upon conditions of the reaction medium such as the concentration of bile salts, ionic strength and the type of the substrate. A pH of 6.5–7.0 was proposed for use with standard pH-stat assay on the basis it was typical of the fasting duodenal pH in healthy subjects. Pancreatin lipase activity was dependent, and was maximal at pH 7-7.5 for LCTs (Corn oil®). The digestion of SCT (TB) and MCT (Miglyol 812®) and mixed glycerides (Imwitor 988®) was less sensitive to change in pH, which may explained by the higher water solubility of C_4 and C_8 fatty acid in acidic conditions.

In general, pancreatic lipase-colipase catalyses the hydrolysis of tri- and diglycerides in the lumen of the small intestine. The enzyme has a preference for sn-1 and sn-3 ester bonds, but displays no activity towards sn-2 linkages. The results are that 2-monoglycerides and fatty acids are the major products of intestinal lipolysis. However, 2- monoglycerides do undergo a slow and non-enzymatic isomerization at alkaline pHs to yield 1- monoglycerides, which are then available for hydrolysis by pancreatic lipase-colipase. The relative proportions of the different triglycerides breakdown products for a healthy humans are typically as follows: glycerol 22%, 2-monoglycerides 72 %, and 1-monoglyceride 6 % (Martin *et al*, 1983).

The effect of increasing calcium concentration from 5mM to 40mM had a negligible impact on the rate of lipolysis of SCTs (TB), MCT (Miglyol 812®) and mixed glycerides (Imwitor 988® or Capmul MCM®). However, the calcium concentration had more pronounced effect on the rate of lipolysis of LCTs (Corn oil®). The lipolysis of long chain triglycerides generated lipolytic products with little solubility (e.g. 10^{-6} and 10^{-3} M] for LC-FAs and MG respectively (Patton *et al*, 1985).

These monoglycerides and fatty acids therefore tend to accumulate at the interface between the digesting oil droplet and the aqueous phase (typically as liquid crystalline bilayers). The clearance of some fatty acid occurred (not MGs) from the surface of a digesting oil droplet through the formation of soaps between one divalent calcium ion and two fatty acids (*i.e.* $Ca^{2+}[FA^{-}]_2$).Bile salts and lecithin are the two major components in bile and the molar ratio is 2:1 to 5:1. Together they are essential to the formation of intestinal mixed micelles. As an amphiphilic compound, lecithin combines with bile salt to form highly solubilizing mixed micelles. The final effect on lipolysis depends on the ratio of lecithin to bile salt; low ratios enhance enzyme activity, whereas high ratios lead to inhibition (Lykidis *et al*, 1997). It is difficult to establish from the literature exactly what is the typical level of BS in the human small intestine. It has been reported that BS concentration ranges from 5.8-37mM (McGregor *et al*, 1997). The average value has been reported as 14.5mM ± 8.8mM. The identity of BS had no appreciable impact on the rate of lipolysis; therefore, in this study inexpensive ox-bile was used instead of NaTDC to determine the effect of concentration of BS on the rate of lipolysis on different substrates.

LCTs (Corn oil®) are dependent on the presence of bile salt micelles or mixed bile salt-lecithin micelles, which can solubilize long-chain fatty acid or monoglycerides. Bile salt concentration had significant effect on the rate of lipolysis of LCTs, but SCTs (TB) were insensitive to bile salt concentration, and MCTs (Miglyol 812®) were much less dependent than LCTs. Similar behaviour was observed for mixed glycerides (Imwitor 988®, Capmul MCM®). This indicates that medium–chain degradation products can be removed from the surface more easily, and did not

require high concentrations of bile salts. Thus, the processing of lipid formulations *in vivo* may depend strongly on the alkyl chain length of TGs used.

A further aspect of SEDDS technology that is generating considerable interest is the role of the surfactant with regard to drug uptake from SEDDS, particularly in terms of the digestibility of the oil. 'Self-microemulsifying' systems which typically contain less oil and more hydrophilic components, such as CRH 40® as well as a co-solvent, may well resist lipolysis during their transit through the gut. The implication of this observation is that such type III formulations are not able to rely on lipolysis to disperse the oil and drug as a colloidal system. To enhance bioavailability they must emulsify efficiently in the stomach by a process independent of but not affected by the gut. Hydrophilic surfactants with hydrophile / lipophilic balance (HLB) values in the range of 13 to 15 were, in general, potent inhibitors of lipase activity towards medium chain triglyceride. Surfactants with dominant lipophilic character were also able to inhibit lipase activity although the inhibition was less severe, with limited lipolysis able to occur from the start of the assay. Non-ionic surfactant, typically trans-esterfication products of TGs and polyoxyethylene, are commonly used in self-emulsifying lipid formulations. Although these surfactants contain fatty acids esters, this study found that hydrolysis of materials in the presence of pancreatin was very limited. Mixtures of oils and hydrophilic surfactants can inhibit lipolysis of triglycerides. Surfactant with HLB > 11 (Cremophor RH 40® / BASF) inhibited lipolysis of TGs and mixed glycerides, if the concentration exceeds 40%w/w surfactant. Hydrophobic surfactant HLB < 10 (Labrafil M1944 Cs®/Gattefossé) did not inhibit lipolysis. Thus, the digestibility of dispersions formed by self-emulsifying systems depends on the surfactants used and the quantity of TG available for lipolysis. The mechanism by which the non-ionic surfactant (HLB >12) inhibit lipolysis that would be occurred once the oxyethylene mantle generated by the surfactant exceeds a critical thickness, which prevents binding of colipase-lipase complex to the surface of the oil droplet.

In general, surfactants which are too hydrophobic to be water-soluble, are poor enhancers, whereas surfactants that are very hydrophilic cannot partition into the hydrophobic environment of the lipid bilayer (Swenson *et al*, 1992). In agreement with these observations, Crison and Amidon (1999) have reported a trend improvement of the bioavailability of nifedipine in dogs using a high HLB surfactant (Labrasol®, HLB 14) over that of a surfactant with low HLB value (Lauroglycol®, HLB 4), even though both formulations appeared to solubilize the drug to the same extent. In this study, a five fold increase in bioavailability with Labrasol® and a three-fold with Lauroglycol® were observed compared to powder formulation.

Solomon *et al* (1996) performed a study of the inhibition of MCT lipolysis by nonyl phenol ethoxylates, which enabled a more systematic study of the influence of HLB on inhibition. The rate of lipolysis was affected by the presence of nonyl phenol ethoxylates with HLB greater than 12, the effect being most pronounced within the HLB range 13-17. At very high HLB the inhibitory effect appeared to decline, an effect which may be explained by the weak surface activity of highly ethoxylated materials. When pure nonyl phenol or ethoxylates of very low HLB were used, the initial rate of hydrolysis was unaffected but the total hydrolysis of fatty acids from MCT oil was lower than expected. This effect could be explained by the phase separation of the oil and alkyl phenol on mixing with water into two populations of droplets (one rich in MCT oil and one in alkyl phenol), only one of which enables lipolysis to proceed (Solomon *et al*, 1996b; MacGregor *et al*, 1997)

Hydrophilic surfactants, orientated at the lipid/water interface may form a hydrophilic barrier around the substrate by means of their ethoxy chains. Initially this barrier would prevent access of colipase to the triglyceride interface, and thus inhibit lipase activity. However, eventually some colipase would be expected to bind followed by lipase, with the consequent production of lipolytic products. Accumulation of these products at the interface may act to promote partitioning of colipase into the substrate surface, with a resultant increase in lipase activity and reduction in the extent of inhibition from the surfactant molecules. Lipophilic surfactants when present in the

system are suggested to speed this process up, either by containing lipolytic products as part of their composition or by providing an additional source of substrate for lipase.

Mono-, di-, and tri-glycerides (Imwitor 988®) and medium chain of fractionated coconut oil (Miglyol 812®) are rapidly digested to free fatty acids and 2-monoglycerides, and these products are solubilized to form a colloidal dispersion within bile salt-lecithin mixed micelles. The essential component of type II system, to promote emulsification of triglycerides or mixed glycerides, is a nonionic ester ethoxylate with intermediate HLB 10-12. These surfactants (*e.g.* polyoxyethylene (25)-glyceryl trioleate [Tagat TO®]) are water dispersible. They tend to form fine stable emulsion. Type II systems will be digestible unless the surfactant (Tagat TO®) concentration is too high. Surfactants themselves with HLB < 11 are very slowly digested and their presence at the oil-water interface can inhibit the digestion of glycerides.TGs present in 'surfactant-free' formulations consisting of oil, co-surfactant and co-solvents were rapidly hydrolyzed. Co-solvents did not appear to influence lipolysis, once the formulation had dispersed. That is thought to be explained by phase separation of oil and co-solvent on dispersion of the formulation.

Porter and Charman (1997) reviewed the use of milk fat globule membrane (MFGM) as an emulsifying agent for the delivery of vitamins (D_3 and A), epidermal growth factor, and insulin (Moriwaki *et al*, 1990). Earlier reports appear to suggest that this emulsifying agent may have some influence on lymphatic uptake, with the recent study suggesting an increase in the lymphatic uptake of vitamin D_3 from emulsion stabilized with MFGM compared to those stabilized with polysorbate-80, although the increase was not statically significant (Liu *et al*, 1995).

The physico-chemical kinetic behaviour of lipid excipients and SEDDS formulations were studied in detail in the presence of the hydrophobic drugs (Chapter 6). The long chain lipid digests separated into an oily phase (containing undigested triglyceride and diglyceride), an aqueous phase (milky thick) (containing bile salt, fatty acid and

monoglyceride) and a pellet phase (containing approximately 5 mm of fatty acid, presumably as an insoluble soap) after ultracentrifugation. Higher proportions of long chain fatty acid and monoglyceride were dispersed into the aqueous phase with increasing bile salt concentrations (Sek *et al*, 2002). In contrast, medium chain lipolytic products separated into a small volume of oily phase (4-6 ml), a precipitate and the rest of the solution, which was clear and/or slightly turbid. The precipitate was believed to be calcium soaps of fatty acids. A pellet fraction could be in a bile-salt-independent manner (Sek *et al*, 2002). On the contrary, the precipitate is calcium dependent-manner. The digestion of both the C_8/C_{10} and C_{18} monoglyceride / diglyceride lipid mixtures was more rapid than the corresponding triglyceride; especially at early time points.

All formulations dispersed to form emulsions in a pure water but their behaviour in simulated intestinal pH-stat solution, was dependent on monoglycerides. When sufficient monoglycerides was present, >60% (Imwitor 988®) we observed demulsification and phase separation, which was dependent on the presence of phospholipid and resulted in sedimentation of what was believed to be a phase rich in monoglycerides swollen with water. The addition of medium chain triglycerides > 30 % (Miglyol 812®) stabilized the formation of mixed micelles, which remained in a finely dispersed state.The results indicate that the micellar hypothesis of fat absorption has been challenged but its general principles have not been changed, although the form in which was presented 20 years ago was an oversimplification.

The floating oil phase contained mostly unhydrolyzed triglycerides and diglycerides. The aqueous phase (micellar) was turbid contained mainly of monoglycerides and fatty acids with small amounts of triglycerides and diglycerides. The aqueous or 'micellar' phase can contain at least two different aggregates: mixed disc-like micelles (multilamellar) saturated with lipolytic product and liposomes (unilamellar vesicles). Usually, in a static system these different phases in micellar phase are in equilibrium and interconvertible. In intestinal content, mixed lipids are formed by lipolysis at the same time as they are mixed with bile. The turbidity and

heterogeneity of micellar phase was due to low total lipid concentration in relation to the bile salt concentration.

In the presence of insufficient bile salt (BL) and phospholipid, the micellar phase may contain liquid crystalline structures and optically isotropic L2 phase (inversed micelles in a lipid bilayer) containing fatty acids and monoglycerides. The presence of L2 is of interest because it consists of a continuous hydrocarbon chain matrix with surface properties resembling those of the oil phase. It may act to help disperse the oil phase, increasing the velocity of lipolysis. In the presence of calcium and insufficient amount of bile salts, 'crusty' crystalline phase (most likely comprising insoluble Ca^{2+}-fatty acid soaps) may form a pellet (precipitate).

In our model system, the unexpected phase which sedimented was a viscous gel-like phase, believed to contain some of monoglycerides and fatty acids. This unexpected phase separation is likely to have a considerable effect on the fate of drug dissolved in such formulations. Mixed mono-, di-, and tri-glycerides are often good solvents for hydrophobic drugs, and are used to increase the solvent capacity of lipid formulations. Our results suggest that use of high concentrations of monoglycerides may be disadvantageous and could result in precipitation of drug. The ability of lipid digestion products to keep the drug in solution is highly dependent on the physico-chemical properties of lipids themselves.

The analysis of hydrophobic drugs (hydrocortisone, testosterone, propyl paraben and methyl paraben) in lipid phases goes hand-on-hand with previous observations. Digestion of mixed mono- di-, and tri-glycerides in the presence of hydrophobic drug under standard pH-stat, suggested that the drug was entrapped in the viscous gel-like phase, thought to be rich in monoglycerides. In the presence of LCT (Corn oil®), more than 65% w/v of the drug remained in the aqueous (micellar) phase. On the contrary, MCT (Miglyol 812®) was only capable of keeping less than 30 % w/v of the drug in solution (chapter 6).

Type III B formulations upon digestion produced a clear aqueous solution which was capable of keeping the steroids in solution due to the presence of (> 40%) hydrophilic surfactant and water soluble co-solvent (> 40 %). In Type III A (<40 %, hydrophilic surfactant and <40 % mixed mono-, di-, and tri-glycerides), ~50 % of the drug was found in the viscous gel-like phase. In the case of surfactant-free formulations (> 50 % mixed mono-, di-, and tri-glycerides, MCT >20% and water-soluble co-solvent < 30%), almost ~ 80% of the drug was found in the subphase. In contrast, Type II formulations supported ~ 45 %w/v of the drug in the micellar phase (subphase) (chapter 6).

7.1 Future work

In general, the new formulation, surfactant-free system can be a promising formulation for lipophilic drugs with high log P >4. Unfortunately, steroids were not the good example used through the research to demonstrate the efficiency of these formulation in terms enhancing their solubility, although, they showed to certain extent an increase in the solubility with high log P >2. Simple solvents of mixtures of mono-, di-, and tri-glycerides can enhance the solubility of steroids better than SEDDS formulation. Because the amount of the steroids dissolved in SEDDS was small, it was difficult to make a clear estimate of the dose that can stay into solution upon dilution.

The validation method of *in vitro* studies of lipolysis using pH-stat method was a descriptive method for the fate of lipid excipients (SCT, MCT, LCT and mixed mono-, di-, and tri-glycerides). It was helpful to make assumption of the fate of SEDDS formulations in terms of how many monoglycerides and fatty acids produced. It was difficult in practice to calculate the MG and FA released from SEDDS formulations. The physical-chemical behaviour of lipid excipients and formulations was a challenge and in the same time difficult. Dealing with the phases in terms of extracting the phases and measuring the concentration of the drug was not an easy process. There could be some errors introduced by practical difficulties in

separation of the phases, leading to error in the estimate of the concentration of the drug dissolved.

In conclusion, there is a need for chronic oral toxicity studies of most common surfactants containing formulation, preferably in gelatin capsule using a suitable animal model. Results from these studies would allow a therapeutically useful window to be identified between absorption enhancing and toxic dose of a particular surfactant. More attention needs to be paid to the characteristics of various lipid formulations available, so that guidelines and experimental methods can be established that allow identification of candidate at an early stage.

It is necessary to establish more methods for detecting the solubilization state of the drug *in vivo*, and there is a need for *in vitro* methods predicting the dynamic changes, which are expected to take place in the gut (*i.e. in vitro / in vivo* correlation methods).

The physical and chemical stability of drugs within lipid systems needs to investigate more and the physical phases formed during lipid digestion may need further studies. The priority for the future should be to conduct human bioavailability studies, and to accomplish more basic studies on the mechanisms of action of this fascinating and diverse group of formulations.

References

Abitec Catalogue, 1995.

Alpers, D.H., Bass, N.M., Engle, M.J., and DeSchryver-Kecskemeti, K. (2000) Intestinal fatty acid binding protein may favor differential apical fatty acid binding in the intestine. *Biochim. Biophys. Acta.* **1483**, 352-362.

Alvarez, F.J., and Stella, V.J. (1989) The role of calcium ions and bile salts on the pancreatic lipase-catalyzed hydrolysis of triglyceride emulsions stabilized with lecithin. *Pharm. Res.* **6**, 449–457.

Amante, A.J., Meier-Kriesche, H.U., Schoenberg, L., and Kahan, B.D. (1997) A pharmacokinetic comparison of the corn oil versus microemulsion gelcap formulation of cyclosporin used *de novo* after renal transplantation. *Transpl. Int.* **10**, 217–222.

Amemiya, T., Mizuno, S., Yuasa, H., and Watanabe, J. (1998) Development of emulsion type new vehicle for soft gelatin capsule. I. selection of surfactants for development of new vehicle and its physicochemical properties. *Chem. Pharm. Bull.* **46**, 309–313.

Amidon, L.G., Leesman, G.D., and Elliot, R.L. (1980) Improving intestinal absorption of water-insoluble compounds: a membrane metabolism strategy. *J. Pharm. Sci.* **69**, 1363–1368.

Amidon, L.G., Lennernas, H., Shah, V.P., and Crison, J.R. (1995) Theoretical basis for a biopharmaceutic drug classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability. *Pharm. Res.* **12**, 413-420.

Anderson, D.B., and Marra, T.M. (1999) Chemical and related factors controlling lipid solubility. Recent advances in the formulation & development of poorly-soluble drugs. *33nd Bull. Tech. Gatt. N° 92*, 11-20. ISSN 0397-7617.

Aoubala, M., de la Fourniere, L., Douchet, I., Abousalham, A., Daniel, C., Hirn, M., Gargouri, Y., Verger, R., and De Caro, A. (1995) Human pancreatic lipase: Importance of the hinge region between the two domains, as revealed by monoclonal antibodies. *J. Biol. Chem.* **270**, 3932-3937.

Armand, M., Borel, P., Ythier, P., Dutot, G., Melin, C., Senft, M., Lafont, H., and Lairon, D. (1992) Effects of droplet size, triacylglycerol composition, and calcium on the hydrolysis of complex emulsions by pancreatic lipase: *in vitro* study. *J. Nutr. Biochem.* **3**, 333-341.

Armand, M., Borel, P., Dubois, C., Senft, M., Peyrot, J., Salducci, J., Lafont, H., and lairon, D. (1994) Characterization of emulsions and lipolysis of dietary lipids in the human stomach. *Am. J. Physiol.* **266**, G372-G381.

Armand, M., Pasquier, B., André, M., Borel, P., Senft, M., Peyrot, J., Salducci, J., Portugal, H., Jaussan, V., and Lairon, D. (1999) Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. *Am. J. Clin. Nutr.* **70**, 1096-1106.

Armstrong, N.A., and James, K.C. (1980) Drug release from lipid-based dosage forms, I and II. *Int. J. Pharm.* **6**, 185-204.

Arnesjö, B., Nilsson, Å., Barrowman, J., and Borgström, B. (1969) Intestinal digestion and absorption of cholesterol and lecithin in the human. Intubation studies with a fat-soluble reference substance. *Scand. J. Gastroent.* **4**, 653–665.

Arnett, E.M., and McKelvey, D.R. (1965) A large solvation enthalpy effect of highly aqueous *t*-butyl alcohol solutions. *J. Am. Chem. Soc.* **87**, 1393-1394.

Arthursson, P., and Karlsson, J. (1991) Correlation between oral drug absorption in humans and apparent drug permeability coefficents in human epithelial (Caco-2) cells. *Biochem. Biophys. Commun.* **175**, 880-885.

Aungst, B.J. (1993) Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolisom. *J. Pharm. Sci.* **82**, 979-987.

Babayan, V.K. (1987) Medium chain triglycerides and structured lipids. *Lipids* **22**, 417–420.

Bachynsky, M.O., Shah, N.H., Patel, C.I., and Malick, A.W. (1997) Factors affecting the efficiency of a self-emulsifying oral delivery system. *Drug Dev. Ind. Pharm.* **23**, 809–816.

Bakatselou, V., Oppenheim, R.C., and Dressman, J.B. (1991) Solubilization and wetting effects of bile salts on the dissolution of steroids. *Pharm. Res.* **8**, 1461-1469.

Barry, B.W., and El-Eini, D.I.D. (1976) Solubilization of hydrocortisone, dexamethasone, testosterone and progesterone by long-chain polyoxyethylene surfactants. *J. Pharm. Pharmacol.* **28**, 210–218.

BASF Technical information, register 5, ME 069 e, (987) August 1998 (MPM).

Bates, T.R., and Sequeira, J.A. (1975) Bioavilability of micronized griseofulvin from corn oil-in-water emulsion, aqueous suspension, and commercial tablet dosage forms in humans. *J. Pharm. Sci.* **64**, 793-797.

Bates, T.R., Gibaldi, M., and Kanig, J.L. (1966) Solubilizing properties of bile salt solutions. II Effect of inorganic electrolyte , lipids, and a mixed bile salt system on solubilization of glutethimide, griseofulvin, and hexestrol. *J. Pharm. Sci.* **55**, 901-906.

Bates, T.R., Lin, S.L., and Gibaldi, M. (1967) Solubilization and rate of dissolution of drugs in the presence of physiologic concentrations of lysolecithin. *J. Pharm. Sci.* **56**, 1492-1495.

Becher, P. (1983a) Encyclopedia of emulsions technology, Vol. 1, Marcel Dekker, Inc., NewYork. pp 342 – 346. ISBN 0-8247-1876-3.

Becher, P. (1983b) Encyclopedia of emulsions technology, Vol. 2, (Applications) Marcel Dekker, Inc., NewYork, pp 613–623. ISBN 0-8247-1877-1.

Becher, P., and Yudenfreund, M.N. (1978) Emulsions, latices, and dispersions. Marcel Dekker, Inc. NewYork. pp 387–394. ISBN 0-8247-6797-7.

Bell, R.M., Ballas, L.M., and Coleman, R.A. (1981) Lipid topogenesis. *J. Lipid Res.* **22**, 391-403.

Bergstedt, S.E., Hayashi, H., Kritchevsky, D., and Tso, P. (1990) A comparison of absorption of glycerol tristearate and glycerol trioleate by rat small intestine. *Am. J. Physiol.* **259**, G386-393.

Bernard, A., Echinard, B., and Carlier, H. (1987) Differential intestinal absorption of absorption of two fatty acid isomers: elaidic and oleic acid. *Am. J. Physiol.* **253**, G751-G759.

Binks, B.P. (1998) Modern aspects of emulsions science. The Royal Society of Chemistry, UK. pp 207–393. ISBN 0-85404-439-6.

Blair, J.A., Lucas, M.L., and Matty, A.J. (1975) Acidification in the rat proximal jejunum. *J. Physiol.* **245**, 333-250.

Bloom, B., Chaikoff, I.L., Reinhardt, W.O., and Dauben, W.G. (1951a) Participation of phospholipides in lymphatic transport of absorbed fatty acids. *J. Biol. Chem.* **189** , 261-267.

Bloom, B., Chaikoff, I.L, Reinhardt, W.O., and Dauben, W.G. (1951b) Intestinal lymph as a pathway for transport of absorbed fatty acids of different chain lengths. *Am. J. Physio.* **166**, 451-455.

Bodmer, M.W., Angal, S., Yarranton, G.T., Harris, T.J., Lyons, A., King, D.J., Pieroni, G., Riviere, C., Verger, R., and Lowe, P.A. (1987) Molecular cloning of a human gastric lipase and expression of the enzyme in yeast. *Biochim. Biophys. Acta.* **25**, 237-244.

Borgström, B. (1954) Effect of tauro-cholic acid on the pH /activity curve of rat pancreatic lipase. *Biochim. Biophys. Acta* **13**, 149-150.

Borgström B., and Erlanson C. (1973) Pancreatic lipase and co-lipase. Interactions and effects of bile salts and other detergents. *Eur. J. Biochem.* **37**, 60-68.

Borgström, B. (1974) Fat digestion and absorption. *Biomembranes J.* **4B**, 555-620.

Borgström, B. (1975) On the interactions between pancreatic lipase and colipase and the substrate and the importance of bile salts. *J. Lipid Res.* **16**, 411-417.

Borgström, B., and Donńer, J. (1975) Binding of bile salts to pancreatic colipase and lipase. *J. Lipid Res.* **16**, 287–292.

Borgström, B. (1976) Binding of pancreatic colipase to interfaces; effects of detergents. *FEBS Lett.* **71**, 201–204.

Borgström, B., and Donńer, J. (1977) The polar interaction between pancreatic lipase, colipase and the triglyceride substrate. *FEBS Lett.* **83**, 23–26.

Borgström, B. (1977a) Physico-chemical characteristics of the lipase-colipase bile salt system. In: Polonvoski J., Cholesterol metabolism and lipolytic enzymes. New York, Masson, pp173-182. ISBN 0893520101.

Borgström, B. (1977b) Digestion and absorption of lipids. *Int. Rev. Physiol.* **12**, 305–323.

Borgström, B. (1977c) The action of bile salts and other detergents on pancreatic lipase and the interaction with colipase. *Biochim. Biophys. Acta* **488**, 381–391.

Borgström, B., Wieloch, T., and Erlanson–Albertsson, C. (1979) Evidence for a pancreatic procolipase and its activation by trypsin. *FEBS Lett.* **108**, 407–410.

Borgström, B. (1980) Importance of phospholipids, pancreatic phospholipase A2, and fatty acid for the digestion of dietary fat. *Gastroenterology* **78**, 954–962.

Borgström, B. (1985) The micellar hypothesis of fat absorption: must it be revisited?. *Scand. J. Gastroenterology.* **20**, 389–394.

Borgström, B., and Erlanson-Albertsson, C. (1984) Pancreatic colipase. In: B. Borgström and H.L. Brockman (Eds.), Lipases. Elesevier, Amsterdam, pp 151-183.

Borgström, B. (1993) Phosphatidylcholine as substrate for human pancreatic phospholipase A2. Importance of the physical state of the substrate. *Lipids* **28**, 371-175.

Bourrel, M., and Schechter, R.S. (1988) Microemulsions and Related Systems (Formulation, Solvency, and Physical Properties), Vol. 30. Marcel Dekker, Inc. NewYork, pp 111–205. ISBN 0-8247-7951-7.

Bowman, W.C., and Rand, M.J. and West, G.B. (1967) Textbook of Pharmacology. Blackwell, London. ISBN 0-632-09990-9.

Breg, J.N., Sarda, L., Cozzone, P.J., Rugani, N., Boelens, R., and Kaptein, R. (1995) Solution structure of porcine pancreatic procolipase as determined from ^{1}H homonuclear two–dimensional and three-dimensional NMR. *Eur. J. Biochem.* **227**, 663–672.

British Pharmacopoeia 2000, The General Medical Council. The Pharmaceutical Press, London. ISBN 0-11-322320.

Brockman, H.L. (1984) General feature of lipolysis :reaction scheme interfacial structure and experimental approaches. In: B. Borgstrom and H.L. Brockman (Eds), Lipases. Elsevier, Amsterdam, pp. 1-46.

Brockerhoff, H. (1971) On the function of bile salts and proteins as cofactors of lipase. *J. Biol. Chem.* **246**, 5828-5831.

Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A., and Thim, L. (1991) A model for interfacial activation of the lipase from the structure of a fungal lipase-inhibitor complex. *Nature* **351**, 491-494.

Brocklehurst, K. (1992) Electrochemical assays: the pH-stat. In: R.Eisenthal and M.J. Danson (Eds), Enzymes Assays: A practical approach. IRL Press, Oxford, pp 191-216. ISBN 0-19-963143-3.

Caliph, S.M., Charman, W.N., and Porter, C.J.H. (2000) Effect of short-, medium- and long-chain fatty acid-based vehicles on the absolute oral bioavailability and intestinal lymphatic transport of halofantrine and assessment of mass balance in lymph-cannulated and non-cannulated rats. *J. Pharm. Sci.* **89**, 1073–1083.

Carey, M.C. (1983) Measurment of physical-chemical properties of bile salt solutions. In Barbara, L., Dowling, R.H., Hofmann, A.F., and Roda, E. (Eds), Bile acids in gastroenterology , MTP Press Boston, pp 19-56. ISBN 0852004885.

Carey, M.C. (1988) Lipid solubilization in bile. In Northfield, T., Jazrawi, R., and Zentler-Munro,P., Bile acids in health and disease. Kluwer lancaster, pp 61–82. ISBN 0746200765

Carey, M.C., Small, D.M. (1970) The characteristics of mixed micellar solution with particular reference to bile. *Am. J. Med.* **49**, 590-608.

Carey, M.C., Small, D.M. (1972) Micelle formation by bile salts. Physical and chemical thermodynamic considerations *Arch. Internal Med.* **130**, 507–527.

Carey, M.C., Small, D.M., and Bliss, C.M. (1983). Lipid Digestion and Absorption. *Ann. Rev. Physiol.* **45**, 651-677.

Challis, D. (1991) Physicochemical and biopharmaceutical studies of novel self-emulsifying systems for administration by the oral route (SEDDS). Ph.D. thesis, University of Bath.

Chang, C., and Bodmeier, R. (1997) Binding of drugs to monoglyceride-based drug delivery systems. *Int. J. Pharm.* **147**, 135–142.

Charman, W.N., and Stella, V.J. (1986) Effect of lipid class and lipid vehicle volume on the intestinal ltmphatic transport of DDT. *Int. J. Pharm.* **33**, 165-172.

Charman , W.N., and Stella, V.J. (1991) Transport of lipophilic molecules by intestinal lymphatic system. *Adv. Drug Del. Rev.***7**, 1-14.

Charman, S.A., Charman, W.N., Rogge, M.C., Wilson, T.D., Dukto, F.J., and Pouton, C.W. (1992) Self-emulsifying drug delivery systems: formulation and biopharmaceutic evalution of an investigational lipophilic compound. *J. Pharm. Res.* **9** , 87–93.

Charman, W.N. Rogge, M.C., Boddy, A.W., and Berger, B.M., (1993) Effect of food and a monoglycerides emulsion formulation on danazol bioavailability. *J. Clin. Pharm.* **33**, 381-386.

Charman, W.N., and Porter C.J.H. (1996). Lipophilic prodrugs designed for intestinal lymphatic transport. *Adv. Drug Del. Rev.***19**, 149-169.

Charman, W.N. (1997) Lipids, lymph and lipdic formulations. *33nd Bull. Tech. Gatt. N° **90***, pp 27-33. ISSN 0397-7617.

Charman, W.N., Porter C.J.H., Mithani, A., and Dressman, J.B. (1997) Physiochemical and physiological mechanisms for the effects of food on drug absorption: the role of lipids and pH. *J. Pharm. Sci.* **86**, 269–282.

Charman, W.N. (1999) Key issues in the development of lipidic formulations. Recent advances in the formulation & development of poorly-soluble drugs. *33nd Bull. Tech. Gatt. N° **92***, pp 9. ISSN 0397-7617.

Charman, W.N. (2000) Lipids, lipophilic drugs, and oral drug delivery-some emerging concepts. *J. Pharm. Sci.* **89**, 967–978.

Cheema, M., Palin, K.J., and Davis, S.S. (1987) Lipid vehicles for intestinal lymphatic drug absorption. *J. Pharm. Pharmcol.* **39**, 55-56.

Chernenko,G.A., Barrowman, J.A., Kean, K.T., Herzberg, G.R., and. Keough, K.M. (1989) Intestinal absorption and lymphatic transport of fish oil (MaxEPA) in the rat. *Biochim. Biophys. Acta* **1004**, 95-102.

Cistola, D.P., Sacchettini, J.C., Banaszak, L.J., Walsh, M.T., and Gordon, J.I. (1989) Fatty acids interaction with rat intestinal and liver fatty acids-binding proteins expressed in *Escherichia coli*, A comparative ^{13}C NMR study. *J. Biol. Chem.* **264**, 2700-2710.

Clark, S.D., and Armstrong, M.K. (1989) Cellular lipid binding proteins: expression, function and nutritional regulation. *FASEB Lett.* **3**, 2480-2487.

Coetzee, J.F., and Ritchie, C.D. (1969) Solute–Solvent interactions. Marcel Dekker Ltd., United Kingdom Edition, pp 321–356. ISBN 69-12718.

Condea Product information, 26.13.202e/09.99.

Condea Product information, 26.13.220e/04.99.

Constantinides, P.P., Scalart, J.P., Lancaster, M.C., Marcello, J., Marks, G., Ellens, H., and Smith, P.L. (1994) Formulation and intestinal absorption enhancement evaluation of water-in-oil microemulsions incorporating medium-chain glycerides. *Pharm. Res.* **11**, 1385–1390.

Constantinides, P.P. (1995) Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. *Pharm. Res.* **12**, 1561-1572.

Constantinides, P.P., Lancaster, M.C., Marcello, J., Chiossone, D.C., Orner, D., Hidalgo, I., Smith, P.L., Sarkahian, A.B., Yiv, S.H., and Owen, A.J. (1995) Enhanced intestinal absorption of an RGD peptide from water-in-oil microemulsions of different composition and particle size. *J. Controlled Release* **34**, 109–116.

Constantinides, P. P., Welzel, G., Ellens, H., Smith, P.L, Sturgis, S., Yiv, S.H., and Owen, A.B. (1996). Water-in-oil microemulsions containing medium-chain fatty acids/salts: Formulations and intestinal absorption enhancement evaluation. *Pharm. Res.*. **13**, 210-215.

Cornish-Bowden, A. (1996) Fundamentals of enzyme kinetics. Academic Press Ltd, United Kingdom. pp 231-299. ISBN 0-408-10617-4.

Cortot, A., Phillips, S.F., and Malagelada, J.R. (1978) Different rates of fat absorption from homogenized and solid meals (Abstract) *Gut*, **19**, A968.

Cortot, A., Phillips, S.F., and Malagelada, J.R. (1982) Parallel gastric emptying of nonhydrolyzable fat and water after a solid-liquid meal in humans. *Gastroenterology* **82**, 877-81.

Couvreur, P., Duchene, D., Kalles, I. (Eds), Prec. Eur.Symp., Formulations of poorly – available drugs for oral administration. Editions de Santé, 17-298, ISBN 2 – 86411 – 096 – 2.

Criag, D.M.Q., Patel, M.J., and Ashford, M. (2000) Administration of emulsions to the gastrointestinal tract. In: Nielloud, F., Marti-Mestres, G., (Eds), Pharmaceutical Emulsions and Suspensions. Marcel Dekker, NewYork. pp 323–360. ISBN 0824703049.

Crison, J.R., and Amidon, G.L. (1999) Method and formulation for increasing the bioavailability of poorly-soluble drugs. US patent, No 5 993 858.

Crounse, R.G. (1961) Human pharmacology of griseofulvin: The effect of Fat intake on the gasterintestinal absorption. *J. Invest. Dermatol.* **37**, 529-533.

Crounse, R.G. (1963) Effective use of griseofulvin. *Arch. Dermatol.* **87**, 176-178.

Curatolo, W., and Ochoa, R. (1994a) Safety assessment of intestinal permeability enhancers. In: A.G. der Boer (Ed.), Drug Absorption Enhancement. Harwood Academic, Switzerland, pp 67–100. ISBN 371865492X.

Curatolo, W., and Ochoa, R. (1994b) Safety assessment of intestinal permeability enhancers. In: A.G. de Boer (Ed.), Drug Absorption Enhancement. Harwood Academic, Switzerland, pp.367-389. ISBN 371865492X.

Davis, A.F., and Hadgraft, J. (1991) Effect of supersaturation on membrane transport: 1. hydrocortisone acetate. *Int. J. Pharm.* **76**, 1–8.

Davis, S.S., Hardy, J.G., and Fara, J.W. (1986) Transit of pharmaceutical dosage forms through the small intestine. *Gut* **27**, 886–892.

Dawson, A.M., and Isselbacher, K.J. (1960) The esterfication of Palmitat–(14) by homogenates of intestinal mucosa. *J. Clin. Invest.* **39**, 150.

del Estal, J.L., Alvarez, A.I., Villaverde, C., and Prieto, J.G. (1993) Comparative effects of anionic, natural bile acid surfactants and mixed micelles on the intestinal absorption of the anthelminitic albendazole. *Int. J. Pharm.* **91**, 105-109.

Dixon, M., Webb, E.C., Thorne, C.J.R. and Tipton, K.F. (1979) Enzymes, 3rd edition. Academic Press Inc., New York. ISBN 0-582-46217-7.

Dressman, J.B., and Fleisher, D. (1986) Mixing tank model for predicating dissolution rate control of oral absorption. *J. Pharm. Sci.* **75**, 109-116.

Dressman, J.B., Amidon, G.L., Reppas, C., and Shah, V.P. (1998) Dissolution testing as a prognostic tool for oral drug absorption: immediate release dosage forms. *Pharm. Res.* **15**, 11–22.

Dressman, J.B., Bass, P., Ritschel, W.A., Friend, D.R., Rubinstein, A., and Ziv, E. (1993) Gastrointestinal parameters that influence oral medications. *J. Pharm. Sci.* **82**, 857–872.

Dressman, J.B., and Reppas,C. (2000) *In vitro-in vivo* correlations for lipophilic, poorly water-soluble drugs. *Eur. J. Pharm. Sci.* **11**, 73-80.

Dutta, S.K., Hamosh, M., Abrams, C.K., Hamosh, P., and Hubbard, V.S. (1982) Quantitative estimation of lingual lipase activity in the upper small intestine in adult patients with pnacreatic insufficiency. *Gastroenterology.* **1047** (Abstr.).

Egelhaaf, S.U., and Schurtenberger, P. (1994) Shape transformations in the lecithin-bile salt system: From cylinders to vesicles. *J. Phys. Chem.* **98**, 8560-8573.

Egloff, M. Marguet, F., Buono, G., Verger, R., Cambillau, C., and van Tillbeurgh, H. (1995) The 2.46 Å resolution structure of pancreatin lipase-colipase complex inhibited by C11 alkyl phosphonate. *Biochem. J.* **34**, 2751-2762

Elworthy, P.H., Florence, A.T., and Macfarlane, C.B. (1975) Solubilization by surface-active agents and its application in chemistry and the biological sciences. Chapman and Hall Ltd., London. pp 13–112. ISBN 0412090309.

Embleton, J.K., and Pouton C.W. (1997). Structure and function of gastro-intestinal lipases. *Adv. Drug Del. Rev.* **25**,15-32.

Eisenthal, R., and Cornish-Bowden, A. (1974) The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. *Biochem. J.* **139**, 715-720.

Eisenthal, R., and Danson, M.J. (1992) Enzyme Assays. A practical approach. IRL Press, Oxford, pp1-300. ISBN 0-19-963143-3.

Erlanson-Albertsson, C. (1992) Pancreatic colipase structural and physiological aspects. *Biochim. Biophys. Acta* **1125**, 1-7.

Farah, N., Laforet, J.P., and Denis, J. (1994) Self-microemulsifying drug delivery systems for improving dissolution of drugs: *in vitro / in vivo* evaluation. *Pharm. Res.* **11**, S-202.

Florence, A.T. (1997) New perspectives in oral delivery: an introduction to the symposium. Recent advances in the formulation & development of poorly-soluble drugs. *31th Bull. Tech. Gatt.*, pp 9-12. ISSN 0397-7617.

Florence, A.T. (1981) Drug Solubilization in Surfactant Systems. In: Techniques of Solubilization of Drugs, edited by Yalkowsky, S.H., Marcel Dekker, INC, NewYork. pp 16–58. ISBN 0-8247-1566-7.

Florence, A.T., and Attwood, D. (1986) Physicochemical principles of pharmacy. Macmillan Education Ltd., Basingstoke. ISBN 0333234049.

Franks, F., and Ives, D.J.G., (1966) The structural properties of alcohol–water mixture. *Photo.Quart. Rev.* **20**, 1-44.

Galia, E., Nicolaides, E., Hörter, D., Löbenberg, R., Reppas, C., and Dressman, J.B. (1998) Evaluation of various dissolution media for predicating *in vivo* perfomance of class I and class II drugs. *Pharm. Res.* **15**, 698–705.

Gargouri, Y., Moreau, H., and Verger, R. (1989) Gastric lipases: biochemical and physiological studies. *Biochim. Biophys. Acta* **1005**, 255-271.

Gargouri. Y., Pieroni, G., Riviere, C., Lowe, P.A., Sauniere, J.F., Sarda, L., and Verger, R. (1986a) Importance of human gastric lipase for intestinal lipolysis: an in vitro study. *Biochim Biophys Acta* **879**, 419-23.

Gargouri, Y., Pieroni, G., Lowe, P.A., Sarda, L., and Verger, R. (1986b) Human gastric lipase: The effect of amphiphiles. *Eur. J. Biochem.* **156**, 305-310.

Gattefossé product information. Gattefossé corporation, Westchester Plaza, Elmsford, New York, data sheet, specification number 3260/00.

Gershanik, T., and Benita, S. (2000) Self-dispersion lipid formulations for improving oral absorption of lipophilic drugs. *Eur. J. Pharm.* **50**, 179–188.

Gershanik, T., Benzeno, S., and Benita, S. (1998) Interaction of a self-emulsifying lipid drug delivery system with the everted rat intestinal mucosa as a function of surface charge and droplet size. *Pharm. Res.* **15**, 863–869.

Granon, S., and Sémériva, M. (1980) Effect of taurodeoxycholate, colipase and temperature on the interfacial inactivation of porcine pancreatic lipase. *Eur. J. Biochem.* **111**, 117–124.

Griffin, W.C. (1949) Calculation of HLB values of non–ionic surfactants. *J. Soc. Cosmet. Chem.* **5**, 249–256.

Grisafe, J.A., and Hayton, W.L. (1978) Effects of short and medium chain fatty acids on absorption of lipophilic drugs from perfused rat intestine. *J. Pharm. Sci.* **67**, 1211-1215.

Groves, M.J. (1978) Spontaneous emulsification. *Chem. Ind.* 417–419.

Groves, M.J., and de Galindez, D.A. (1976) The self – emulsifying action of mixed surfactants in oil. *Acta Pharm. Suec.* **13**, 361–372.

Gupta, S.K., Manfro, R.C., Tomlanovich, S.J., Gambertoglio, J.G., Garovoy, M.R., and Benet, L.Z. (1990) Effect of food on the pharmacokinetics of cyclosporine in healthy subjects following oral and intravenous administration. *J. Clini. Pharm.* **30**, 643-653.

Gurr, M.I., and James, A.T. (1971) Lipid Biochemistry: an introduction. Chapman and Hall Ltd., London. ISBN 0412101106.

Hamaguchi, T., Shinkuma, T., Irie, T., Yamanka, Y., Morita,Y., Iwamoto, B., Miyoshi, K., and Mizuno, N. (1993) Effect of high-fat meal on the bioavilability of phentoyin in commercial powder with a large particle size. *Int. J. Clin. Pharm. Ther. Tox.* **31**, 326-330.

Hamosh, M. (1984) Lingual lipase. In:B. Borgstom and H.I.Brockman (Eds.), lipases. Elsevier, Amesterdam, pp. 49-81. ISBN 0444805265.

Hauss, D.J, Fogal, S.E., Ficorill, J.V., Price, C.A., Roy, T., Jayaraj, A.A., and Keirns, J.J. (1998). Lipid-based delivery systems for improving the bioavilablity and lymphatic transport of a poorly water-soluble LTB_4 inhibitor. *J. Pharm. Sci.* **87**, 164-169.

Hedeman, H., Brøndsted, H., Müllertz, A., and Frokjaer, S. (1996) Fat emulsions based on structured lipids (1,3-specific triglycerides): an investigation of the *in vivo* fate. *Pharm. Res.* **13**, 725–728.

Heertjes, P.M., and Witvoet, W.C. (1969/1970) Some aspects of the wetting of powders. *Powder Tech.* **3**, 339-343.

Henderson, P.J.F (1992) Statistical analysis of enzyme kinetic data. In: R.Eisenthal and M.J. Danson (Eds), Enzymes Assays: A practical approach. IRL Press, Oxford, pp 276-314. ISBN 0-19-963143-3.

Hermoso, J., Pignol, D., Kerfelec, B., Crenon, I., Chapus, C., and Fontecilla-Camps, J.C. (1996) Lipase activation by non-ionic detergents. The crystal structure of the porcine lipase-colipase-tetraethylene glycol monooctyl ether complex. *J. Biol. Chem.* **271**, 18007–18016.

Hernell, O., Staggers, J.E., and Carey, M.C. (1990) Physical-chemical behavior of dietry and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochem. J.* **29**, 2041-2056.

Hjelm, R.P., Thiyagarajan, P., and Alkan-Onyuksel, H. (1992) Organization of phosphatidylcholine and bile salt in rodlike mixed micelle. *J. Phys. Chem.* **96**, 8653-8661.

Hofmann, A.F., and Borgström, B. (1964) The intraluminal phase of fat digestion in man: the lipid content of the micellar and oil phases of intestinal content obtained during fat digestion and absorption. *J. Clin. Invest.* **43**, 247-257.

Hofmann, A.F. (1963) The behavior and solubility of monoglycerides in dilute, micellar bile-salt solution. *Biochem. Biophys. Acta* **70**, 306–316.

Hofmann, A.F., and Borgström, B. (1963) Hydrolysis of long-chain monoglycerides in micellar solution by pancreatic lipase. *Biochem. Biophys. Acta* **70**, 317–331.

Hofmann, A.F., and Mysels, K.J. (1992) Bile acid solubility and precipitation *in vitro and in vivo*: the role of conjugation, pH, and Ca^{2+} ions. *J. Lipid Res.* **33**, 617–627.

Hoffman, N.E., and Hofmann, A.F. (1973) A comparison of the rate of absorption of micellar and nonmicellar oleic acid. *Digestive Dis.* **18**, 489–492.

Hoffman, N.E. (1970) The relationship between uptake *in vitro* of oleic acid and micellar solubilization. *Biochim. Biophys. Acta* **196**, 193-203.

Holmberg, I., Aksnes, L., Berlin, T., Lindback, B., Zemgals, J. and Lindeke, B. (1990) Absorption of a pharmacological dose of vitamin D3 from two different lipid vehicles in man: comparison of peanut oil and a medium chain triglyceride. *Biopharm. Drug Dispos.* **11**, 807–815.

Hörter, D., and Dressman, J.B. (2001) Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. *Adv. Drug Del. Rev.* **46**, 75–87.

Hultin, M., Müllertz, A., Zundel, M.A., Olivercona, G., Hansen, T.T., Deckelbaum, R.J., Carpentier, Y.A., and Olivercona, T. (1994) Metabolisom of emulsions containing medium- and long-chain triglycerides or interesterified triglycerides. *J. Lipid Res.* **35**, 1850–1860.

Humberstone, A.J., and Charman, W.N. (1997) Lipid-based vehicles for the oral delivery of poorly water soluble drugs. *Adv. Drug Del. Rev* **25**, 103-128.

Hutchison, K.G., MacGregor, K., Embleton, J.K., Seager, H., Solomon, L.J., and Pouton, C.W. (1996) Optimizing the gastro–intestinal absorption of hydrophobic drugs using micro–emulsions and other liquid formulations. In: Couvreur, P., Duchene, D., Kalles, I. (Eds), Proc. Eur. Symp., *Formulations of poorly–available drugs for oral administration.* Editions de Santé, 121–125, ISBN 2–86411–096–2.

Israelachvili, J.N. (1992) Intermolecular and Surface Forces. Academic Press LTD, UK. pp 344–347. ISBN 0-12-375181-0.

Jain, N., Yang, G., Tabibi, S.E., and Yalkowsky, S.H. (2001) Solubilization of NSC-639829. *Int. J. Pharm.* **225**, 41–48.

James, K.C. (1986) Solubility and Related Properties, Marcel Dekker, Inc. NewYork, pp 95–115. ISBN 0-8247-7484-1.

Johnston, J.M., and Borgstrom, B. (1964) The intestinal absorption and metabolism of micellar solutions of lipids. *Biochem. Biophys. Acta* **84**, 412–423.

Kabalnov, A., Tarara, T., Arlauskas, R., and Weers, J. (1996) Phospholipids as Emulsion Stabilizers. *J. Colloid Interface Sci.* **184**, 227-35.

Khoo, S., Humberstone, A., Porter, C.J.H., Edwards, G.A., and Charman, W.N. (1998) Formulation design and bioavailability assessment of lipidic self-emulsifying formulations of halofantrine. *Int. J. Pharm.* **167**, 155–164.

Khoo, S., Porter, C.H.J., and Charman, W.N. (2000) The formulation of halofantrine as either non-solubilising PEG 6000 or solubilising lipid-based solid dispersions: physical stability and absolute bioavailability assessment. *Int. J. Pharm.* **205**, 65-78.

Kimura, F., Murakami, S., Fujishira, R. (1975) Thermodynamic of aqueous solutions of nonelectrolytes. II, Enthalpies of transfer of 1-methyl-2-pyrrolidinone from water to many aqueous alcohols. *J. Sol. Chem.* **4,** 241-247.

Klyashchitsky, B.A., and Owen, A.J. (1998) Drug delivery systems for cyclosporin : achievements and complications. *J. Drug Target.* **5**, 443–458.

Kommuru, T.R., Gurley, B., Khan, M.A., and Reddy, I.K. (2001) Self-emulsifying drug delivery systems (SEDDS) of coenzyme Q_{10}: formulation development and bioavailability assessment. *Int. J. Pharm.* **212**, 233–246.

Kozolv, M.M., Lichtenberg, D., and Andelman, D. (1997) Shape of phospholipid / Surfactant mixed micelles: Cylinders or disks? Theortical analysis. *J. Phys. Chem. B* **101**, 6600-6606.

Kratohvil, J.P., and Dellicolli, H.T. (1970) Measurment of the size of micelles: the case of sodium taurodeoxycholate. *Photo. Fed. Proc.* **29**, 1335-1342.

Kwei, G.Y., Novak, L.B., Hettrick, L.H., Reiss, E.R., Fong, E.K., Olah, T.V., and Loper, A.E. (1998) Lymphatic uptake of MK-386, a sterol 5-alpha reductase inhibitor, from aqueous and lipid formulations. *Int. J. Pharm.*. **164**, 37-44.

Larsson, A., and Erlanson-Albertsson, C. (1981) The identity and properties of two forms of activated colipase from porcine pancreas. *Biochim. Biophys. Acta* **664**, 538-548.

Larrson, A., and Erlanson-Albertsson, C. (1986) Effect of phosphatidylcholine and free fatty acids on the activity of pancreatic lipase-colipase. *Biochim. Biophys. Acta* **876**, 543-550.

Larrson, A., and Erlanson-Albertsson, C. (1991) The effect of pancreatic procolipase and colipase on pancreatic activation. *Biochim. Biophys. Acta* **1083**, 283-288.

Lawrence, A.S.C., and Durham, K. (1961) Surface activity and detergency, McMillan & Co., London, 152-159.

Lawrence, M.J. (1996) Microemulsions as drug delivery vehicles. *Photo. Curr. Opin. Colloid. Interface. Sci.* **1**, 826–832.

Lawrence, M.J., Rees, G.D. (2000). Microemulsion-based media as novel drug delivery systems. *Adv. Drug Del. Rev.* **45**, 89-121.

Lennernäs, H. (1998) Human intestinal permeability. *J. Pharm. Sci.* **87**, 403–410.

Levitt, M.D., Strocchi, A., and Levitt, D.G. (1992) Human jejunal unstirred layer: evidence for extremely efficient luminal stirring. *Am. J. Physiol.* **262**, G593-G596.

Lewis, M.C., and Root, C. (1990) *In vivo* transport kinetics and distribution of taurocholate by rat ileum and jejunum. *Am. J. Physiol.* **259**, G233-G238.

Li, C., Zimmerman, C.L., and Weidmann, T.S. (1996) Solubilization of retinoids by bile salt / phospholipid aggregates. *Pharm. Res.* **11**, 907–913.

Li, P., Tabibi, E., and Yalkowsky, S.H. (1999) Solubilization of ionized and un-ionized flavopiridol by ethanol and polysorbate 20. *J. Pharm. Sci.* 88, 507–509.

Liao, T.H., Hamosh, P., and Hamosh, M. (1984) Fat digestion by lingual lipase: mechanism of lipolysis in the stomach and upper small intestine. *Ped. Res.* **18**, 403–409.

Lievens, and Hildegard, S.R. (1992) Pharmaceutical formulations of benzodiazepines. *European Patent Application* EP 0 517 412 A1, 26.

Lindmark, T., Nikkila, T., and Arthursson, P. (1995) Mechanisms of absorption enhancement by medium chain fatty acids in intestinal epithelial Caco-2 monolayers. *J. Pharmacol. and Exp. Ther.* **275**, 958-964.

Lipinski, C.A., Lombardo, F., Dominy, B., and Feeney, P.J. (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Del. Rev.* **23**, 3–25.

Lissant, K.J. (1974) Emulsions and Emulsions Technology, Marcel Dekker, Inc. New York, Part 1, pp 44–48. ISBN 0-8247-6097-2.

Liu, H-X., Adachi, I., Horikoshi, I., and Ueno, M. (1995) Mechanism of promotion of lymphatic drug absorption by milk fat globule membrane. *Int. J. Pharm.*, **118**, 55-64.

Liversidge, G.G., and Cundy, K.C. (1995) Particle size reduction for improvement of oral bioavailability of hydrophobic drugs: I. Absolute oral bioavailability of nanocrystalline danazol in beagle dogs. *Int. J. Pharm.* **125**, 91-97.

Löbenberg, R., and Amidon, G.L. (2000) Modern bioavailability, bioequivalence and biopharmaceutics classification system. New scientific approaches to international regulatory standards. *Eur. J. Pharm. Biophys.* **50**, 3-12.

Loper, A.E., Booth, S., Clarke, A., Olah, T.V., McLoughlin, D.A., Novak, L.B., Hettrick, L.A., and Storey, D.E. (1996) Equivalence of a self-emulsifying drug delivery system (SEDDS) and soybean oil for oral delivery of a 5α-reductase inhibitor in rhesus monkeys. In: Couvreur, P., Duchene, D., Kalles, I. (Eds), Prec. Eur.Symp., Formulations of poorly – available drugs for oral administration. Editions de Santé, 369-372, ISBN 2 – 86411 – 096 – 2.

Lucas, M.L. (1984) Pharmacology of intestinal premeation. II., Csaky, T.Z. (Ed.), Berlin: Springer-Verlag, pp. 119-163. ISBN 0387131019.

Luthi-Peng, Q., Marki, H.P., and Hadvary, P. (1992) Identification of the active site serine in human pancreatic lipase by chemical modification with tetrahydrolipstatin. *FEBS Lett.* **299**, 111-115.

Lykidis, A., Avranas, A., and Aszoglou, P. (1997) Combined effect of a lecithin and a bile salt on pancreatic lipase activity. *Comp. Biochem. Physiol.* **116B**, 51–55.

MacGregor, K.J., Embleton, J.K., Lacy, J.E., Perry, E.A., Solomon, L.J., Seafer, H., and Pouton, C.W. (1997) Influence of lipolysis on drug absorption from the gastro-intestinal tract. *Adv. Drug Del. Rev.* **25**, 33-46.

Malcolmson, C., and Lawrence, M.J. (1993) A comparison of the incoporation of model into non-ionic micellar and microemulsions. *J. Pharm. Pharmacol.* **45**, 141–143.

Malcolmson, C., Satra, C., Kantaria, S., Sidhu, A., and Lawrence, M.J. (1998) Effect of oil on the level of solubilization of testosterone propionate into nonionic oil-in-water microemulsions. *J. Pharm. Sci.* **87**, 109–116.

Mansbach, C.M., Cohen, R.S., and Leff, P.B. (1975) Isolation and properties of the mixed lipid micelles present in intestinal content during fat digestion in man. *J. Clin. Invest.* 56, 781-791.

Marszall, L. (1987) HLB of nonionic surfactants: PIT and EIP methods. In Schick, M.J. (Eds.) Nonionic Surfactants: Physical chemistry volume 23. Marcel Dekker Inc., New York, pp 493-547. ISBN 0824775309.

Martha, A.G., Omar, S.M., and Kassem, M.A. (1982) Study of the influence of sodium taurocholate and sodium glycocholate on mass transfer of certain drugs: Diethylstillbestrol. *Int. J. Pharm.* **11**, 27-34.

Martigne, M., Julien, R., and Sarda, L. (1987) Studies on the effect of bile and lipolysis products on pancreatic lipase and colipase activity *in vitro*. *Reprod. Nutr. Dévelop.* **27**, 1005–1012.

Martin, D.W., Mayes, P.A., and Rodwell, V.W. (1983) Harper's Review of Biochemistry, 19th Edition, Lange Medical Publications, California, USA, Chapter 41, pp. 546-558. ISBN 0870410377.

Martindale: The Extra Pharmacopoeia, 30th revision. (1993) J.E.F. Reynolds (Eds.), The Pharmaceutical Press, London. ISBN 0-85369-300-5.

Mazer, N.A., Benedek, G.B., and Carey, M.C. (1980) Quasielastic light-scattering studies of aqueous biliary lipid systems. Mixed micelle formation in bile salt-lecithin solutions. *Biochem. J.* **19**, 601-615.

McBain, M.E.L., and Hutchinson, E. (1955) Solubilization and related phenomena. Academic Press Inc., New York.

McBain, J.W., Merrill, R.C., and Vinograd, J.R. (1941) The solubilization of water-insoluble dye in diute solutions of aqueous detergents. *J. Am. Chem. Soc.* **63**, 670.

McDonald, G.B., Saunders, D.R., Weidman, M., and Fisher, L. (1980) Portal venous transport of long chain fatty acids absorbed from small intestine. *Am. J. Physiol.* **239**, G141-150.

McDonald, G.B., and Weidman, M. (1987) Partitioning of polar fatty acids into lymph and portal vein after intestinal absorption in the rat. *Quart. J. Exp. Physiol.* **72**, 153-159.

Meli, A., Cargill, D.I., Giannina, T., and Steinetz, B.G. (1968) Studies on the transport of estrogens by the rat small intestine *in vivo*. *Proc. Soc. Exp. Biol. Med.* **129**, 937-944.

Meyrs, R.A., and Stella, V.J. (1992) Systemic bioavailabililty of penclomidine (NSC-338720) from oil-in-water emulsions administered intraduodenally to rats. *Int. J. Pharm.* **78**, 217-226.

Miller, C.A., and Neogi, P. (1985) Interfacial phenomena (Equilbrium and dynamic effects). Marcel Dekker, Inc. NewYork, pp 160-170. ISBN 0-8247-7490-6.

Mithani, S.D., Bakatselou, V., TenHoor, C.N., and Dressman, J.B. (1996) Estimation of increase in solubility as a function of bile salt concentration. *Pharm. Res.* **13**, 163-167.

Mittal, K.L. (1977) Micellization, Solubilization, and Microemulsions. Vol. 1, Plenum Press, NewYork, pp 1–89. ISBN 0-306-31023-6.

Mittal, K.L. (1977) Micellization, Solubilization, and Microemulsions. Vol. 2, Plenum Press, NewYork, pp 538–539. ISBN 0-306-31024-4.

Miyazaki, S., Inoue, T., Yamahira, T., and Nadai, T. (1979) Interactions of drugs with bile components.I. Effects of bile salts on the dissolution behaviour of indomethacine and pheylbutazone. *Chem. Pharm. Bull.* **27**, 2468-2472.

Miyazaki, S., Inoue, T., Yamahira, T., and Nadai, T. (1981) Micellar interaction of indomethacin and phenylbutazone with bile salts. *Int. J. Pharm.* **8**, 303-310.

Moreau, H., Bernadac, A., Gargouri, Y., Benkouka, F., Laugier, R., and Verger, R. (1989) Immunocytolocalization of human gastric lipase in chief cells of the fundic mucosa. *Histochem. J.* 91, 419-423.

Morelock, L.M., Choi, L.L., Bell, G.L., and Wright, J.L. (1994) Estimation and correlation of drug water solubility with pharmacological parameters required for biological activity. *J. Pharm. Sci.* **83**, 948–952.

Moriwaki, T., Yoshikwa, H., Takada, K., and Muranishi, S. (1990) DDS with MFGM(milk fat globule membrane): gastrointestinal absorption of vitamin A acetate. Yakuzaikaku **50**, 263-267.

Morre, D.J. (1977) Golgi apparatus and membrane biogenesis. *Cell Surface Rev.* **4**, 1-83.

Morris, J.G. (1974) A biologist' physical chemistry. 2nd edition by Edward Arnolod (Ltd), London. pp 249-321. ISBN 0-7131-2414-8.

Mueller, E.A., Kovarik, J.M., VanBree, J.B., Grevel, J., and Kutz, K. (1994a) Influence of a fat-rich meal on the pharmacokinetics of a new oral formulation of cyclosporin in a crossover comparison with the market formulation. *Pharm. Res.* **11**, 151-155.

Mueller, E.A., Kovarik, J.M., VanBree, J.B., Grevel, J., Tetzloff, W., and Kutz, K. (1994b) Improved dose linearity of cyclosporin pharmacokinetics from a microemulsion formulation. *Pharm. Res.* **11**, 301-304.

Muranishi, S., and Yamamoto, A. (1994) Mechanisms of absorption enhancement through gastrointestinal epithelium. In: A.G. de Boer (Ed), Drug Absorption Enhacement . Harwood Academic, Switzerland, pp. 67-100. ISBN 371865492X.

Muranishi, S. (1985) Modification of intestinal absorption of drugs by lipoidal adjuvants. *Pharm. Res.* **2**, 108-118.

Muranushi, N., Kimugawa, M., Nakajima, Y., Muranishi, S., and Sezaki, H. (1980). Mechanism for the inducement of the intestinal absorption of poorly absorbed drugs by mixed micelles I. Effect of various lipid bile salt mixed micelles on the absorption of streptomycin in rat. *Int. J. Pharm.* **4**, 271-279.

Nankervis, R., Davis, S.S., Day, N.H., and Shaw, P.N. (1996) Intestinal lymphatic transport of three retinoids in the rat after oral administration: effect of lipophilicity and lipid vehicle. *Int. J. Pharm.* **130**, 57–64.

Narayanan, V.S., and Stroch, J. (1996) Fatty-acid transfer in taurodeoxycholate mixed micelles. *Biochem. J.* **25**, 7466-7473.

Naylor, L.J., Bakatselou, V., Rodriguez-Hornedo, H., Weiner, N.D., and Dressman, D. (1995) Dissolution of steroids in bile salt solutions is modified by the presence of lecithin. *Eur. J. Pharm. Biophys.* **41**, 346-353.

Naylor, L.J.M. (1993) Dissolution mechanisms of poorly soluble compounds in simple and mixed micelle systems. Ph.D Thesis, University of Michigan.

Nerurkar, M.M., Burton, P.S., and Borchardt, R.T. (1996). The use of surfactants to enhance permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system. *Pharm. Res.* **13**, 528-534.

Nicolaides, E., Galia, E., Efthymiopoulos, C., Dressman, J.B., and Reppas, C. (1999) Forecasting the *in vivo* performance of four low solubility drugs from their *in vitro* dissolution data. *Pharm. Res.* **16**, 1876–1882.

Noguchi, T., Charman, W.N.A., and Stella V.J. (1985) The effect of drug lipophilicity and lipid vehicles on the lymphatic absorption of various testosterone esters. *Int. J. Pharm.* **24**, 173-184.

Nördskog, B.K., Phan, C.T., Nutting, D.F., and Tso, P. (2001) An examination of the factors affecting intestinal lymphatics transport of dietary lipids. *Adv. Drug Del. Rev.* **50**, 21-44.

Nunn, W.D., Collburn, R.W., and Black, P.N. (1986) Transport of long-chain fatty acids in Escherichia coli. Evidence for role of fadL gene product as long-chain fatty acid receptor. *J. Biol. Chem.* **261**, 167-171.

O'Connor, J., and Wallace, R.G. (1984) Studies in bile salt solutions. The effect of pH on the cholate and taurocholate stimulation of human milk lipase catalyzed hydrolysis of *p*-nitrophenyl acetate. *Eur. J. Biochem.* **141**, 379-383.

O'Driscoll, C.M. (1996) Micellar solubilization (Self-emulsifying Systems for improved Absorption of Drugs). In: Couvreur, P., Duchene, D., Kalles, I. (Eds), Prec. Eur.Symp., Formulations of poorly – available drugs for oral administration. Editions de Santé, 126–139, ISBN 2 – 86411 – 096 – 2.

O'Driscoll, C.M., Obodozie, O.G., and Corrigan, O.I. (1994) The solubility and rat intestinal absorption of DDT in mixed micellar systems. *Eur. J. Drug Metab. Pharm.* **35**, 74–78.

O'Driscoll, C.M., Reilly, J.R., and Corrigan, O.I. (1991) A comparison of the effect of synthetic and naturally occurring surfactants on the solubility and absorption of clofazimine (B 663). *Eur. J. Drug Metab. Pharmacol.* **3**, 116-119.

Ockner, R.K., and Manning, J.A. (1974) Fatty acid-binding protein in small intestine: idetification, isolation and evidence for its role in cellular fatty acids transport. *J. Clin. Invest.* **54**, 326-338.

Ockner, R.K., Pittman, J.P., and Yager, J.L. (1972) Differences in the intestinal absorption of saturated and unsaturated long chain fatty acids. *Gastro. J.* **62**, 981-992.

Oliver, R.E., Jones, A.F., and Rowland, M. (1998) What surface of the intestinal epithelium available to permeating drugs? *J. Pharm. Sci.* **87**, 634–639.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussman, J.L., Verschueren, K.H.G., and Goldman, A. (1992). The alpha / beta hydrolase fold. *Protein Eng.* **5**, 197-211

Palin, K.J., and Wilason, C.G. (1984) The effects of different oils on the absorption of prpbuocl in the rat. *J. Pharm. Pharmacol.* **36**, 641-643.

Palin, K.J., Phillips, A.J., and Ning, A. (1986). The oral absorption of cefoxitin from oil and emulsion vehicles in rats. *Int. J. Pharm.* **33**, 99-104.

Palin, K.J., Wilson , C.G., Davis, S.S., and Phillips A.J. (1982) The effect of oils on the lymphatic absoption of DDT. *J. Pharm. Pharmacol.* **34**, 707-710.

Patton, J.S., Albertsson, P.A., Erlanson, C., and Borgström, B. (1978) Binding porcine pancreatic lipase and colipase in the absence of substrate studied by two-phase partition and affinity chromatography. *J. Biol. Chem.* **253**, 4195-4202.

Patton, J.S., and Carey, M.C. (1979) Watching fat digestion. *Science* **204**, 145-148.

Patton, J.S., and Carey, M.C. (1981) Inhibition of human pancreatic lipase-colipase activity by mixed bile salt-phospholipid micelles. *Am. J. Physiol.* **241**, G328-336.

Pedersen, J.S., Egelhaaf, S.U., and Schurtenberger, P. (1995) Formation of polymer like mixed micelles and vesicles in lecithin-bile salt solutions: Small-angle neutron-scattering study. *J. Phys. Chem.* **99**, 1299-1305.

Piéroni, G., Gargouri, Y., Sarda, L., and Verger, R. (1990) Interactions of lipases with monolayers. Facts and questions. *Adv. Colloid Interface Sci.* **32**, 341-378.

Poelma, F.G.J., Breäs, R., Tukker, J.J., and Crommelin, D.J.A. (1991). Intestinal absorption of drugs. The influence of mixed micelles on the disapperance kinetics of drugs from the small intestine of the rat. *J. Pharm. Pharmacol.* **43**, 317-324.

Porter, C.J.H. (1997) Drug delivery to the lymphatic system. *Crit. Rev. Ther. Drug Carrier Systems* **14**, 333–393.

Porter, C.J.H. (1999) Lipids, gastrointestinal uptake and drug absorption: *In vivo* and *in vitro* model selection. Recent advances in the formulation & development of poorly-soluble drugs. *33nd Bull. Tech. Gatt.* 21-30. ISSN 0397-7617.

Porter, C.J.H., and Charman, W.N. (2001a) *In vitro* assessment of oral lipid based formulations. *Adv. Drug Del. Rev.* **50**, 127-147.

Porter, C.J.H., and Charman, W.N. (2001b) Intestinal lymphatic drug transport: an update. *Adv. Drug Del. Rev.* **50**, 61-80.

Porter, C.J.H., and Charman, W.N. (1997) Uptake of drugs into the intestinal lymphatics after oral administration. *Adv. Drug Del. Rev.* **25**, 71-89.

Porter, C.J.H., Charman, S.A., Humberstone, A.J., and Charman, W.N. (1996) Lymphatic transport of halofantrine in the conscious rat when administered as either the free base or the hydrochloride salt: effect of lipid class and lipid vehicle dispersion. *J. Pharm. Sci.* **85** , 357-361.

Porter, H.P., and Saunders, D.R. (1971) Isolation of the aqueous phase of human intestinal contents during the digestion of a fatty meal. *Gastroen. J.* **60**, 997–1007.

Pouton, C.W. (1997) Formulation of self-emulsifying drug delivery systems. *Adv. Drug Del. Rev.* **25**, 47–58.

Pouton, C.W. (1999) Key issues when formulating hydrophobic drugs with lipids. Recent advances in the formulation & development of poorly-soluble Drugs. *33nd Bull. Tech. Gatt.* N° **92**, pp 41-50. ISSN 0397-7617.

Pouton, C.W. (2000) Lipid formulation for oral administration of drugs: non–emulsifying, self–emulsifying and 'self-microemulsifying' drug delivery systems. *Eur. J. Pharm. Sci.* **11**, 593–598.

Pouton, C.W. (1982) A study of self–emulsifying oil/surfactant mixutres. Ph.D thesis, University of London.

Pouton, C.W., and Charman, W.N. (1997) The potential of oily formulations for drug delivery to the gastro-intestinal tract. *Adv. Drug Del. Rev.* **25**, 1–2.

Ran, Y., Zhao, L., Xu, Q., and Yalkowsky, S.H. (2001) Solubilization of cyclosporin A. *AAPS Pharm. Sci. Tech.* **2**, 1–8.

Renner, F., Samuelson, A., Rogers, M., and Glickman, R.M. (1986) Effect of saturated and unsaturated lipid on the composition of mesenteric triglyceride-rich lipoproteins in the rat. *J. Lipid Res.*, **27**, 72-81.

Robb, I.D. (1982) Microemulsions, Plenum Press, NewYork and London, pp 186–187. ISBN 0-306-40834-1.

Robino, J.T., and Yalkowsky, S.H. (1987a) Cosolvency and cosolvent polarity. *Pharm. Res.* **4**, 220–230.

Robino, J.T., and Yalkowsky, S.H. (1987b) Cosolvency and deviations from log-linear solubilization. *Pharm. Res.* **4**, 231–236.

Roman, R. (1999) So you want to use lipid–based formulations in development. *33nd Bull. Tech. Gatt.* N° **92**, pp 51-58. ISSN 0397-7616.

Rosoff , M., and Serajuddin, A.T.M. (1980) Solubilization of diazepam in bile salts and in sodium cholate-lecithin-water phases. *Inter. J. Pharm.* 6, 137-146.

Rubino, J.T., and Obeng, E.K. (1991) Influence of solute structure on deviations from the log-linear solubility equation in propylene glycol: water mixtures. *J. Pharm. Sci.* **80**, 479-483.

Samaha, M.W., and Naggar, V.F. (1988) Micellar properties of non-ionic surfactants in relation to their solubility parameters. *Int. J. Pharm.* **42**, 1–9.

Sarciaux, J.M., Acar, L., and Sado, P.A. (1995) Using microemulsion formulations for oral drug delivery of theraputic peptides. *Int. J. Pharm.* **120**, 127–136.

Saunders, L. (1971) Principles of Physical Chemistry for Biology and Pharmacy. Oxford University Press, London. ISBN 0198597053.

Schwizer, W., Borovicka, J., Kunz, P., Fraser, R., Kreiss, C., D'Amato, M., Crelier, G., Boesiger, P., and Fried, M. (1997) Role of cholecystokinin in the

regulation of liquid gastric emptying and gastric motility in humans: studies with the CCK antagonist loxiglumide. *Gut* **41**, 500-504.

Schwarz, M.A., Neubert, R.H.H., and Dongowski, G. (1996). Characterization of interaction between bile salts and drugs by micellar electrokinetic capillary chromatography. Part I. *Pharm. Res.* **13**, 8, 1174-1180.

Segel, I.H. (1975) Enzyme kinetics. Behaviour and analysis of rapid equilbrium and steady-state enzyme systems. Wiley-Interscience Inc., Toronto. ISBN 0471774251.

Sek, L., Porter, C.J.H., Kaukonen, A.M., and Charman, W.N. (2002) Evaluation of the *in vitro* digestion profiles of long and medium chain glycerides and the phase behaviour of their lipolytic products. *J. Pharm. Pharmacol.* **54**, 29–41.

Semeriva, M., Dufour, C., and Desneuelle, P. (1971) On the probable involvement of a histadine residue in the active site of pancreatic lipase. *Biochem. J.* **10**, 2143-2149.

Shah, N. H., Carvajal, M.T., Patel, C.I., Infeld, M.H., and Malick, A.W. (1994). Self-emulsifying drug delivery systems (SEDDS) with polyglycolyzed glycerides for improving *in vitro* dissolution and oral absorption of lipophilic drugs. *Int. J. Pharm.* **106**, 15-23.

Shankland, W. (1970) The equilbrium and structure of lecithin-cholate mixed micelles. *Chem. Phys. Lipids* **4**, 109–130.

Shiau, Y.F. (1981) Mechanisms of intestinal fat absorption. *Am. J. Physiol.* **240**, G1-G9.

Shiau, Y.F. (1990) Mechanism of intestinal fatty acid uptake in the rat: the role of an acidic microclimate. *J. Physiol.* **239**, G177-G182.

Shiau, Y.F., Popper, D.A., Reed, M., Umstetter, C., Capuzzi, D., and Levine, G. (1985) Intestinal triglycerides are derived from both endogenous and exogenous sources. *Am. J. Physiol.* **148**, G164-G169.

Shinoda, k., and Becher, P. (1978) Principles of Solutions and Solubility. Marcel Dekker, Inc. NewYork, pp 164–209. ISBN 0-8247-6717-9.

Small, D.M. (1967) Physicochemical studies of Cholesterol gallstone formation. *Gaster. J.* **52**, 607-610.

Smith, A., and Lough, A.K. (1976) Micellar solubilization of fatty acids in aqueous media containing bile salts and phospholipids. *Br. J. Nutr.* **35**, 77-87.

Solomon, L. (1998) Self-emulsifying drug delivery systems (SEDDs). Ph.D.thesis University of Bath.

Solomon, L.J., Embleton, J.K., and Pouton, C.W. (1996a) Solubilization of steroidal compounds by mixed bile salt–lecithin micelles. In: Couvreur, P., Duchene, D., Kalles, I. (Eds), Prec. Eur.Symp., *Formulations of poorly–available drugs for oral administration.* Editions de Santé, Paris, 219–222, ISBN 2 – 86411 – 096 – 2.

Solomon, L.J., Embleton, J.K., and Pouton, C.W. (1996b) Inhibtion of lipolysis of medium- chain triglycerides by non-ionic surfactants, a structure / activity study. In: Couvreur, P., Duchene, D., Kalles, I. (Eds), Prec. Eur. Symp., *Formulations of poorly–available drugs for oral administration.* Editions de Santé, Paris, 437-438, ISBN 2 – 86411 – 096 – 2.

Stadler, P., Kovac, A., Haalck, L., Spener, F., and Paltatuf, F. (1995) Stereoselectivity of microbial lipases: The substitution at position sn-2 of triacyglycerol analogs influences the stereoselectivity of different microbial lipases. *Eur. J. Biochem.* **227**, 335-343.

Staggers, J.E. , Hernell, O., Stafford, R.J., and Carey, M.C. (1990) Physical–chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase behavior and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. *Biochem. J.* **29**, 2028-2040.

Staggers, J.E., Fernando-Warnakulasuriya, G.J.P., and Wells, M.A. (1981). Studies on fat digestion, absorption and transport in the suckling rat. II. Triacylglycerols; molecular species, stereospecific analysis and specificity of hydrolysis by lingual lipase. *J. Lipid Res.* **22**, 675-679.

Stevens, R.W., and Green, C. (1972) The effect of side chain structure on the incorporation of steroids into lipid bilayers (liposomes). *FEBS Lett.* **27**,145-148.

Stremmel, W., Lotz, G., Strohmeyer, G., and Berk, P.D. (1985) Identification, isolation and partial characterization of a fatty acid binding protein from rat jejunal microvillus membranes. *J. Clin. Invest.* **75**, 1068-1076.

Swenson, E.S., and Curatolo, W.J. (1992). Means to enhance penetration intestinal permeability enhancement for proteins, peptides and other polar drugs: mechanisms and potential toxicity. *Adv. Drug Del. Rev.* **8**, 39-92.

Sylven, A., and Borgström, B. (1969) Intestinal absorption and lymphatic transport of cholestrol in the rat: influence of the fatty acid chain length of the carrier triglyceride. *J. Lipid Res.* **10**, 351-355.

Taylor, M.D. (1996) Improved passive oral drug delivery via prodrugs. *Adv. Drug Del. Rev.* **19**, 131–148.

The Merck Index 12th edition. (1996) S. Budavari, (Ed.), Merck & Co. Inc., White house station, New Jersey. ISBN 0911910-12-3.

Thomson, A.B., Dietshy, J.M., and Csaky, T.Z., Eds. (1984) International pharmacology of intestinal permeation II; Berlin: Springer-Verlag. ISBN 3540131019.

Thomson, A.B., Schoeller, C., Keelan, M., Smith, L., and Clandinin, M.T. (1993) Lipid absorption: passing through the unstirred layers, brush-border membrane, and beyond. *Can. J. Physiol. Pharmacol.* **71**, 531-555.

Toothacker, R.D., and Welling, P.G. (1980) The effect of food on drug bioavailability. *Annu .Rev. Pharmacol. Toxicol.* 20, 173-199.

Thumser, A.E., and Storch, J. (2000) Liver and intestinal fatty acid-binding proteins obtain fatty acids from phospholipid membranes by different mechanisms. *J. Lipid Res.* **41**, 647-656.

Tipton, K.F. (1992) Principles of enzyme assay and kinetic studies. In: R.Eisenthal and M.J. Danson (Eds), Enzyme assays: A practical approach. IRL Press, Oxford, 1-58. ISBN 0-19-963143-3.

Trenktrog, T., Müller, B.W., and Seifert, J. (1995) *In vitro*-investigation into the enhancement of intestinal peptide absorption by emulsion system. *Eur. J. Pharm. Biopharm.* **41**, 284–290.

Triuppathi, C., and Balasubramanian, K.A. (1982) Purification and properties of an acid lipase from human gastric juice. *Biochim. Biophys. Acta* **712**, 692-697.

Tso, P. (1985) Gastrointestinal digestion and absorption of lipid. *Adv. Lipid Res.* **21**, 143-286.

Tso, P., Buch, K.L., Balint, J.A., and Rodgers, J.B. (1982) Maximal lymphatic triglycerides transport rate from the rat small intestine. *Am. J. Physiol.* **242**, G408-415.

Tso, P., Karlstad, M.D., Bistrian, B.R., and DeMichele, S.J. (1995) Intestinal digestion, absorption, and transport of structured triglycerides and cholesterol in rats. *Am. J. Phys.* **268**, G568-577.

Tso, P., Lee, T., and Demichele, S.J. (1999) Lymphatic absorption of structured triglycerides *vs* physical mix in a rat model of fat malabsorption. *Am. J. Physiol.* **277**, G333-340.

Tso, P., Lindstrom, M.B., and Borgström, B. (1987) Factors regulating the formation of chylomicrons and very-low-density lipoproteins by the rat small intestine. *Biochim. Biophys. Acta* **922**, 304-313.

United States Pharmacopiea & National Formulary (2000) USP-24, NF-19, 24rd revision. United States Pharmacopeial Convention Inc., Rockville. ISBN 0195-7996.

Vahouny, G., and Treadwell, C.R. (1958) Comparative effects of dietary fatty acids and triglycerides on lymph lipids in the rat. *Am. J. Physio.* **196**, 881-883.

Vandermeers, A., Vandermeers-Piret, M.C., Rathe, J., and Christophe, J. (1974) On human pancreatic triacylglycerol lipase: isolation and some properties. *Biochim. Biophys. Acta* **370**, 257-268.

van Tilbeurgh, H., Egloff, M., Martinez, C., Rugani, N., Verger, R., and Cambillau, C. (1993) Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography. *Nature* **362**, 814-820.

Verger, R., Mieras, M.C., and de Haas, G.H. (1973) Action of phospholipase A at interfaces. *J. Biol. Chem.* **248**, 4023-4034.

Verger, R., Rietsch, J., and Desnuelle, P. (1977) Effects of colipase on hydrolysis of monomolecular films by lipase. *J. Biol. Chem.* **252**, 4319–4325.

Verger, R. (1984) Pancreatic lipase. In: Borgström, B., and Brockman, H.L., Lipases. Elsevier ; New York, pp 83-150. ISBN 0444805265.

Vonderscher, J., and Meinzer, A. (1994) Rational for the development of Sandimmune Neoral. *Transplant Proc.* **26**, 2925–2927.

Wakerly, M.G., Pouton, C.W., Meakin, B.J., and Morton, F.S. (1986) Self-emulsification of vegetable oil-nonionic surfactant mixtures. *Am. Chem. Soc. Symp. Ser.* **311**, 242-255.

Walters. K.A., Dugard, P.H., and Florence, A.T. (1981) Non-ionic surfactants and gastric mucosal transport of paraquat. *J. Pharm. Pharmacol.* **33**, 207-213.

Walter, A., Vinson, P.K., Kaplun, A., and Talmon, Y. (1991) Intermediate structures in the cholate-phosphatidylcholine vesicle-micelle transition. *Biophys. J.* **60**, 1315-1325.

Weintraub, H., and Gibaldi, M. (1969) Physiologic surface-active agents and drug absorption. IV. Effects of premicellar concentrations of surfactant on dissolution rate. *J. Pharm. Sci.* **58**, 1368-1372.

Westergaard, H., and Dietschy, J. (1974) Delineation of the dimensions and permeability characteristics of the two major diffusion barriers to passive mucosal uptake in the rabbit intestine. *J. Clin. Invest.* **174**, 718-732.

Wickham, M., Garrood, M., Leney, J., Wilson, P.D.G., and Fillery-Travis, A. (1998) Modification of a phospholipid stabilized emulsion interface by bile salt: effect on pancreatic lipase activity. *J. Lipid Res.* **39**, 623-632.

Wieloch, T., Borgström, B., Piérone, G., Pattus, F., and Verger, R. (1982) Product activation of pancreatic lipase. Product activation of pancreatic lipase. Lipolytic enzymes as probes for lipid /water interfaces. *J. Biol. Chem.* **257**, 11523–11528.

Williams, N.A., and Amidon, G.L. (1988) The estimation of solubility in binary solvents: application of the reduced 3-suffix solubility equation to ethanol-water mixtures. *Pharm. Res.* **5**, 193–195.

Winkler, F.K., D'Arcy, A., and Hunziker, W. (1990) Structure of human pancreatic lipase. *Nature* **343**, 771-774.

Winstanely, P.A., and Orme, M.L.E. (1989) The effects of food on drug bioavailability. *Br. J. Clin. Pharm.* **28**, 621-628.

Yalkowsky, S.H. (1999) Solubility and Solubilization in aqueous media. New York, NY: University Press. ISBN 0841235767.

Yalkowsky, S.H., and Roseman, T.J. (1981) Solubilization of drugs by cosolvents. In: Techniques of solubilization of drugs, edited by Yalkowsky, S.H., Marcel Dekker, Inc., NewYork. pp 92–134. ISBN 0-8247-1566-7.

Yang, Y., and Lowe, M.E. (2000) The open mediates pancreatic lipase function. *J. Lipid Res.* **41**, 48–57.

Yeh, P., Smith, P.L., and Ellens, H. (1994) Effect of medium-chain glycerides on physiological properties of rabbit intestinal epithilium *in vitro*. *Pharm. Res.* **11**, 1148–1154.

Yu, L., Bridgers, A., Polli, J., Vickers, A., Lang, S., Roy, A., Winnike, R., and Coffin, M. (1999) Vitamin E-TPGS increases absorption flux of an HIV protease inhibitor by enhancing its solubility and permeability. *Pharm. Res.* **16**, 1812-1817.

Yu, L.X., Lipka, E., Crison, J.R., and Amidon, G.L. (1996) Transport approaches to the biopharmaceutical design of oral drug delivery systems: predication of intestinal absorption. *Adv. Drug Del. Rev.* **19**, 359–376.

Zhao, D., and Hirst, B.H. (1990) Comparison of bile salt perturbation of duodenal and jejunal isolated brush-border membranes. *Digestion J.* **47**, 200–207.

Appendix 1

Steroids	λmax Theoretical	λmax Practical	$E_{cm}^{1\%}$ Theoretical	$E_{cm}^{1\%}$ Practical	CV
Hydrocortisone	241.5*	242	445**	438.74	2.26
Hydrocortisone acetate	241.5*	241	390**	387.12	0.71
Progesterone	240**	240	314.47**	514.02	0.64
Testosterone	240*	240	288.43 **	550.16	3.31
Testosterone acetate	241*	241		517.02	0.13

* from British Pharmacopoeia
** from Merck index

Table 1.1a Represents the parameters (λmax and $E_{cm}^{1\%}$) used for the calculation of solubility (g % w/v) of steroids in chapters 2 and 3 using the UV spectrophotometer method.

Hydroxy benzoate derivatives	λmax Theoretical	λmax Practical	$E_{cm}^{1\%}$ Theoretical	$E_{cm}^{1\%}$ Practical	CV
Butyl paraben	295*	252**	840	874	0.92
Methyl paraben	261*	231**		296.18	0.67
Ethyl paraben	295*	235**		457	3.44
Propyl paraben	296*	266**		180	0.67

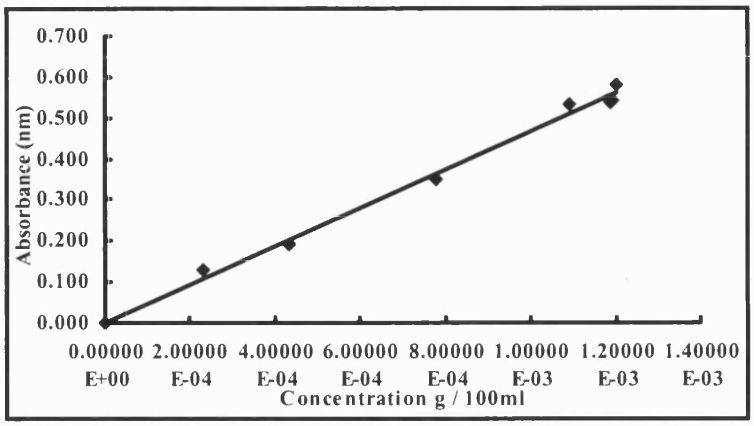
* Merck index where diluted using alcohol 96 % v/v
** The solvent used was Acetonitrile 99 % v/v

Table 1.1b Represents the parameters (λmax and $E_{cm}^{1\%}$) used for the calculation of solubility (g % w/v) of hydroxy benzoate derivatives in chapter 2 using the UV spectrophotometer method.

Hydrocortisone standard curve

A series of hydrocortisone solutions was prepared by dilution with ethanol 96 % v/v. Samples were produced which contained (0.432, 0.780,1.09, 1.191 and 1.188) mg % of hydrocortisone. An analysis was performed as described in 2.2.2.2. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

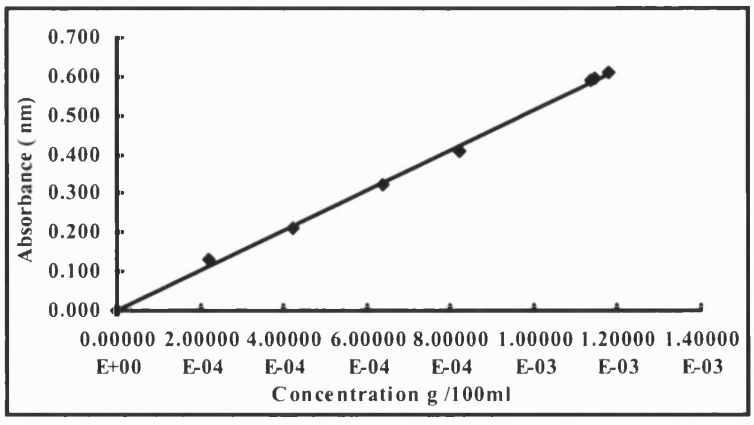
Slope = 466.06X $\quad$ $\mathbf{r^2}$ = 0.9941 $\quad$ Intercept = 0



Progesterone standard curve

A series of progesterone solutions were prepared by dilution with ethanol 96 % v/v. Samples were produced which contained (0.431, 0.640, 0.821, 1.136, 1.145 and 1.180) mg % of hydrocortisone. An analysis was performed as described in 2.2.2.2. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

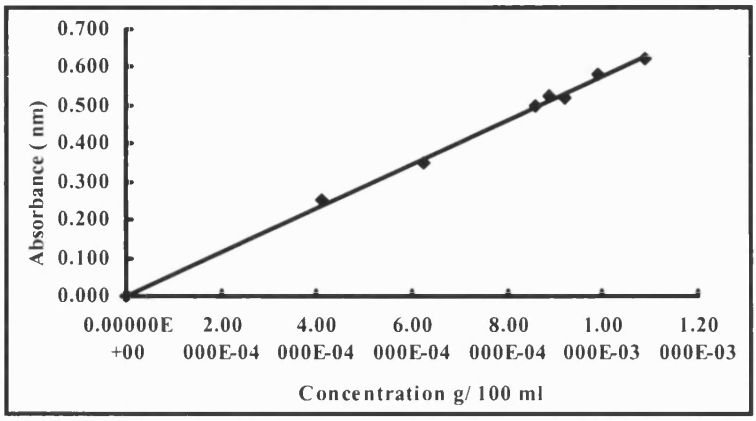
Slope = 513.5X $\quad$ $\mathbf{r^2}$ = 0.9985 $\quad$ Intercept = 0



Testosterone standard curve

A series of testosterone solutions were prepared by dilution with ethanol 96 % v/v. Samples were produced which contained (0.412, 0.860, 0.920, 0.999 and 1.09) mg % of testosterone. An analysis was performed as described in 2.2.2.2. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

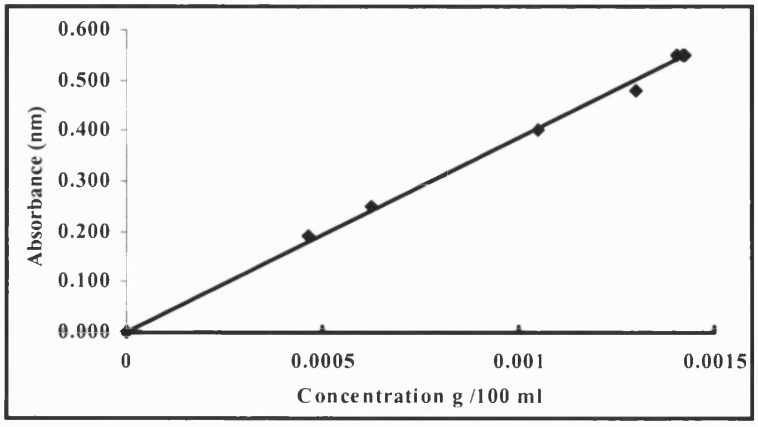
Slope = 578.28X $\quad$ $\mathbf{r^2}$ = 0.9976 $\quad$ Intercept = 0



Testosterone acetate standard curve

A series of testosterone acetate solutions were prepared by dilution with methanol 96 % v/v. Samples were produced which contained (0.412, 0.830, 1.1408, 1.1456 and 1.168) mg % of testosterone acetate. An analysis was performed as described in 2.2.2.2. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

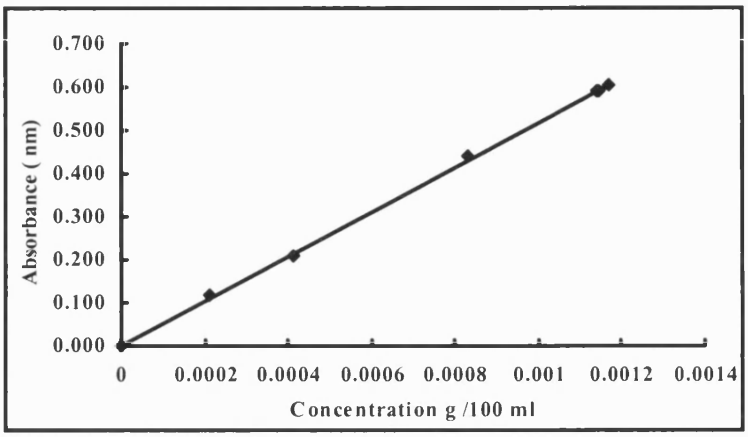
Slope = 519.1X $\quad$ $\mathbf{r^2}$ = 0.9994 $\quad$ Intercept = 0



Hydrocortisone acetate standard curve

A series of hydrocortisone acetate solutions were prepared by dilution with methanol 96 % v/v. Samples were produced which contained (0.467, 0.623, 1.050, 1.300 and 1.426) mg % of hydrocortisone acetate. An analysis was performed as described in 2.2.2.2. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

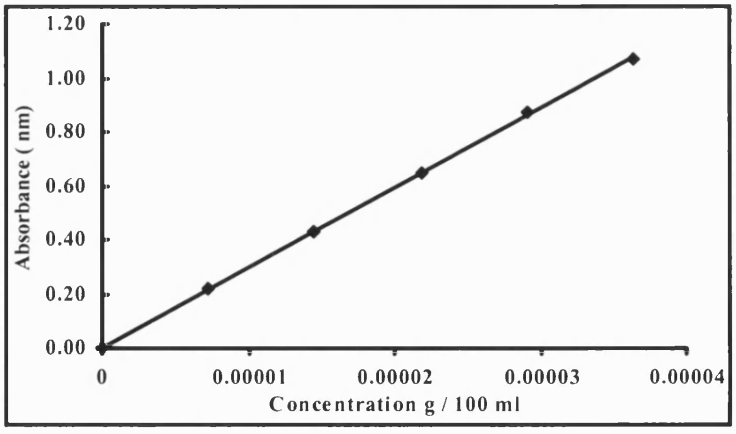
Slope = 384.29X $\quad$ $\mathbf{r^2}$ = 0.998 $\quad$ Intercept = 0



Methyl paraben standard curve

A series of methyl paraben solutions were prepared by dilution with acetonitrile 96 % v/v. Samples were produced which contained (0.02912, 0.02184, 0.01450, 0.364 and 0.0728) mg % of ethyl paraben. An analysis was performed as described in 2.2.2.2. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

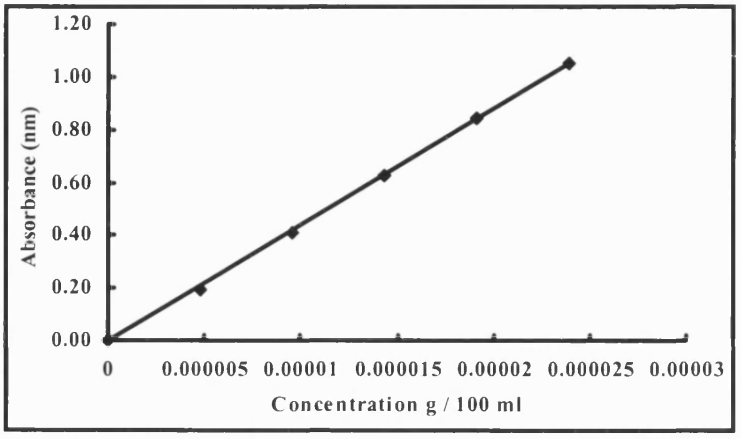
Slope = 29610X $\quad$ $\mathbf{r^2}$ = 0.9998 $\quad$ Intercept = 0



Ethyl paraben standard curve

A series of ethyl paraben solutions were prepared by dilution with acetonitrile 96 % v/v. Samples were produced which contained (0.014352, 0.01936, 0.04784, 0.02392 and 0.09568) mg % ethyl paraben. An analysis was performed as described in 2.2.2.2. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

Slope = 43824X $\quad$ $\mathbf{r^2}$ = 0.9995 $\quad$ Intercept = 0



Propyl paraben standard curve

A series of propyl paraben solutions were prepared by dilution with acetonitrile 96 % v/v. Samples were produced which contained (0.01168, 0.02393, 0.03598, 0.04786 and 0.05982) mg % propyl paraben. An analysis was performed as described in 2.2.2.2. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

Slope = 18018X $\quad$ $\mathbf{r^2}$ = 0.9998 $\quad$ Intercept = 0

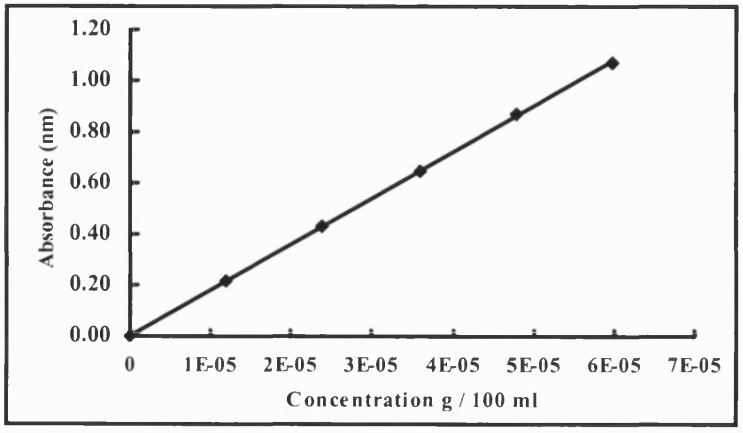


Table 1.2a *Percentage (w/v) solubility(S_i) of steroids in lipid excipients at 25 °C.*

	Imwitor 988®			Capmul MCM®			Miglyol 812®			*Cremophor RH 40®			Tagat TO®		
Drug (Solubility % w/v) (Si)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)
Hydrocortisone	1.55	0.04	1.85	1.002	0.034	3.344	0.100	0.003	3.054	0.73	0.007	0.955	0.1	0.006	5.864
Hydrocortisone acetate	0.22	0.007	3.24	0.122	0.07	5.27	0.386	0.008	2.156	0.15	0.004	2.98	0.025	0.002	8.221
Progesterone	1.60	0.03	2.83	1.2	0.019	1.58	1.147	0.024	2.064	1.21	0.033	2.734	0.83	0.034	7.203
Testosterone	0.82	0.004	0.449	0.79	0.015	1.93	0.847	0.033	3.849	0.97	0.068	7.011	0.301	0.013	4.381
Testosterone acetate	1.10	0.012	1.066	1.04	0.053	5.11	1.035	0.012	1.166	1.06	0.03	2.846	1.274	0.018	2.548

*Cremophor RH 40® solubility were determined at 30°C.

Table 1.2b *Percentage (w/v) solubility (S_i) of steroids in lipid excipients at 25 °C.*

	Propylene glycol			PEG400			Transcutol P®		
Drug (Solubility % w/v) (Si)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)
Hydrocortisone	1.00	0.013	1.300	1.228	0.039	3.202	1.077	0.064	5.91
Hydrocortisone acetate	0.367	0.021	5.816	0.333	0.001	0.204	0.78	0.038	5.37
Progesterone	1.071	0.007	1.064	1.171	0.028	2.396	1.037	0.023	2.18
Testosterone	0.822	0.018	2.18	0.95	0.008	0.832	1.11	0.009	0.76
Testosterone acetate	0.876	0.019	5.303	0.92	0.019	2.055	1.142	0.008	0.71

Table 1.3 *Percentage (w/v) solubility (Si) of steroids in S-F formulations at 25 °C.*

Drug %w/v (Si)	I988®+M812®+(PG+Gly) 50 %+ 30 %+ 20 % Mean (n=3)	SD (n=3)	RSD (n=3)	I988®+M812®+Trans P® 50 %+ 30 %+ 20 % Mean (n=3)	SD (n=3)	RSD (n=3)	I988®+M812®+ PG 50 %+ 30 %+ 20 % Mean (n=3)	SD (n=3)	RSD (n=3)	I988®+M812®+PEG 50 %+30 %+ 20 % Mean (n=3)	SD (n=3)	RSD (n=3)
H.	0.6331	0.019	0.926	0.614	0.006	0.963	1.203	0.018	1.927	0.747	0.0122	1.631
H.A	0.1144	0.002	1.4017	0.178	0.0018	1.054	0.1632	0.0024	1.4904	0.162	0.005	2.857
P.	2.376	0.019	0.959	2.598	0.009	0.756	2.215	0.0193	0.851	2.06	0.101	4.225
T.	1.5488	0.023	1.47	0.926	0.0125	1.345	2.576	0.0811	4.571	1.373	0.030	1.926
T.A	2.018	0.009	1.349	1.111	0.0141	1.27	2.06	0.0157	0.8513	1.850	0.024	1.422

Table 1.4 *Percentage (w/v) solubility (S_i) of steroids in type II formulations at 25 °C.*

Miglyol 812® :Tagat To®	Hydrocortisone % w/v S_i			Testosterone % w/v S_i			Testosterone Acetate % w/v S_i			Hydrocortisone Acetate % w/v S_i			Progesterone % w/v S_i		
Ratio	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)
20: 80	0.418	0.008	1.980	0.504	0.023	4.464	0.459	0.032	6.950	0.01	0.001	2.410	1.057	0.117	1.104
30 :70	0.391	0.013	4.570	0.625	0.007	1.139	0.510	0.008	1.472	0.017	0.001	4.490	1.049	0.009	0.858
40 : 60	0.290	0.016	5.470	0.674	0.027	2.937	0.562	0.029	3.510	0.029	0.001	1.540	1.00	0.004	0.265
50 : 50	0.21	0.029	3.514	0.721	0.017	2.342	0.612	0.020	1.487	0.03	0.002	1.544	0.98	0.024	1.99
60 : 40	0.176	0.002	1.366	0.772	0.027	2.303	0.636	0.015	0.822	0.048	0.001	4.770	0.89	0.030	2.406
70 : 30	0.151	0.006	0.822	0.791	0.020	2.510	0.669	0.006	5.270	0.059	0.001	2.266	0.86	0.038	2.834
80 : 20	0.105	0.003	2.930	0.82	0.008	1.308	0.690	0.034	3.560	0.069	0.002	3.200	0.81	0.016	1.624

Table 1.5 *Percentage (w/v) solubility (S_i) of steroids in type III A at 25 °C.*

Imwitor 988®+ Miglyol 812® + Cremophor RH 40®+ PEG400	Hydrocortisone g % w/v S_i			Hydrocortisone acetate g % w/v S_i			Progesterone g% w/v S_i			Testosterone g % w/v S_i			Testosterone Acetate g % w/v S_i		
Ratio	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)
20%+ 30 %+ 30 %+ 20 %	0.705	0.003	0.350	0.194	0.001	0.006	1.266	0.031	2.590	0.956	0.025	2.620	1.037	0.206	1.980
20 %+ 30 %+25 %+25 %	0.763	0.005	0.666	0.206	0.004	1.716	1.312	0.002	0.170	0.928	0.012	1.240	1.080	0.053	4.860
15 %+35 %+ 30 %+ 20 %	0.713	0.005	0.675	0.173	0.006	0.369	1.263	0.033	2.810	0.850	0.025	2.880	0.904	0.003	0.272
35 %+ 15 %+ 25 %+25 %	0.777	0.010	1.240	0.193	0.002	1.237	1.163	0.010	0.827	0.910	0.015	1.637	1.044	0.009	0.184
25 %+25 %+30 %+20 %	0.739	0.028	3.735	0.193	0.009	4.890	1.364	0.006	0.422	1.034	0.022	2.140	1.104	0.020	1.800

Table 1.6 *Percentage (w/v) solubility (S_i) of steroids in type IIIB at 25 °C.*

Imwitor 988®+Miglyol 812® + Cremophor RH 40®+PEG 400	Hydrocortisone g % w/v S_i			Hydrocortisone acetate g % w/v S_i			Progesterone g % w/v S_i			Testosterone g % w/v S_i			Testosterone Acetate g % w/v S_i		
Ratio	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)
5 %+15 %+40 %+ 40 %	1.009	0.003	0.318	0.230	0.002	0.723	1.489	0.008	0.510	1.130	0.002	0.161	0.840	0.003	0.320
10 %+10 %+ 40 %+ 40 %	1.089	0.009	0.811	0.300	0.001	0.269	1.305	0.002	0.147	1.096	0.0004	0.035	0.798	0.003	0.403

Table 1.7 *Percentage (w/v) solubility (Si) of mixture of mono-, di-, and tri-glycerides and medium chain fatty acids oil at 25 °C.*

Miglyol 812® : Imwitor 988®	Hydrocortisone g % w/v S_i			Testosterone g % w/v S_i			Testosterone Acetate g % w/v S_i			Hydrocortisone Acetate g % w/v S			Progesterone g % w/v S_i		
Ratio	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)
30 :70	0.506	0.009	1.752	0.921	0.015	1.314	1.253	0.022	1.779	0.117	0.005	4.436	1.433	0.018	1.369
40 : 60	0.436	0.011	2.478	0.917	0.013	1.206	1.194	0.021	1.729	0.099	0.006	6.522	1.341	0.021	1.695
50 :50	0.379	0.006	1.463	0.909	0.007	0.550	1.157	0.041	3.839	0.072	0.005	5.318	1.249	0.017	1.364
60 :40	0.325	0.015	4.690	0.90	0.033	2.900	1.02	0.004	0.404	0.062	0.0035	3.692	1.214	0.011	0.920
70 : 30	0.244	0.006	2.446	0.892	0.016	1.490	0.93	0.016	1.383	0.054	0.0052	0.001	1.176	0.052	3.593
80 : 20	0.152	0.006	3.988	0.838	0.013	1.416	0.84	0.022	0.279	0.042	0.001 3	0.005	1.049	0.011	

Table 1.8 *Percentage (w/v) solubility (S_i) of steroids in mixture of mono-, di-, and tri-glycerides and propylene glycol as hydrophilic co-solvent at 25 °C.*

I 988® : PG	Hydrocortisone g % w/v S_i			Testosterone g % w/v S_i			Testosterone Acetate g % w/v S_i			Hydrocortisone Acetate g % w/v S_i			Progesterone g % w/v Si		
Ratio	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)
30 :70	1.871	0.019	0.980	0.962	0.403	1.863	0.796	0.013	0.773	0.239	0.007	2.719	1.68	0.027	1.118
40 : 60	1.830	0.022	1.1830	1.98	0.063	3.500	0.718	0.054	3.139	0.241	0.003	1.162	1.52	0.024	0.928
50 :50	1.759	0.020	1.092	2.35	0.132	3.002	0.838	0.030	3.730	0.251	0.005	1.780	1.39	0.003	0.118
60 :40	1.662	0.028	1.692	2.149	0.087	2.858	0.862	0.006	0.836	0.240	0.017	7.120	1.21	0.062	2.434
70 : 30	1.470	0.025	1.666	1.737	0.050	4.061	0.943	0.143	6.276	0.233	0.001	0.601	1.10	0.042	1.87
80 : 20	1.242	0.020	1.586	1.755	0.053	5.607	0.965	0.061	3.106	0.212	0.007	3.448	1.01	0.019	0.803

Table 1.9 *Percentage (w/v) solubility (S_i) of steroids in a mixture of mono-, di-, and tri-glycerides and polyethylene glycol as hydrophilic co-solvent at 25 °C.*

Imwitor 988® : PEG 400	Hydrocortisone g % w/v S_i			Testosterone g % w/v S_i			Testosterone Acetate g % w/v S_i			Hydrocortisone Acetate g % w/v S_i			Progesterone g % w/v S_i		
Ratio	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)
30 :70	1.630	0.012	0.708	0.897	0.091	5.177	1.19	0.030	2.428	0.337	0.002	0.603	1.90	0.031	1.563
40 : 60	1.587	0.009	0.521	0.884	0.064	3.559	1.1	0.011	0.731	0.329	0.001	0.315	1.81	0.047	2.440
50 :50	1.486	0.011	0.758	0.871	0.056	3.230	1.04	0.056	3.790	0.325	0.007	2.233	1.69	0.071	3.099
60 :40	1.367	0.039	2.837	0.859	0.090	2.658	1.0	0.005	0.353	0.321	0.005	1.945	1.57	0.118	5.239
70 : 30	1.264	0.0075	0.596	0.841	0.069	7.650	0.97	0.046	2.973	0.319	0.008	3.361	1.38	0.175	6.850
80 : 20	1.148	0.040	3.470	0.834	0.026	3.061	0.93	0.047	2.744	0.316	0.007	3.099	1.21	0.115	4.425

Table 1.10 *Effect of increasing temperature Vs time intervals on the solubility (S_i) % w/v profile of testosterone in S-F formulations.*

Time	S_i % w/v		S_i % w/v		S_i % w/v		S_i % w/v		S_i % w/v		S_i % w/v		S_i % w/v	
hour	25°C	SD	30°C	SD	40°C	SD	50°C	SD	60°C	SD	70°C	SD	80°C	SD
0	1.046	0.0052	3.1440	0.0045	3.5060	0.0084	3.873	0.0067	5.2267	0.0027	3.9880	0.0847	4.7400	0.0100
6	2.240	0.0031	3.2650	0.0032	3.6100	0.0056	3.336	0.0053	4.5900	0.0063	4.1670	0.0092	4.1837	0.0095
12	2.349	0.0039	3.3850	0.0041	3.1200	0.0034	3.360	0.0028	3.5190	0.0073	3.6220	0.0094	3.8870	0.0043
24	2.450	0.0062	3.0600	0.0046	2.8710	0.0076	3.220	0.0027	3.3930	0.0039	3.3860	0.0052	3.5600	0.0093
36	2.56	0.0041	2.8370	0.0036	2.9190	0.0029	3.090	0.0082	3.2200	0.0024	3.3100	0.0024	3.3500	0.0089
48	2.591	0.0049	2.8370	0.0090	2.9000	0.0087	2.9170	0.0051	3.1700	0.0023	3.1100	0.0095	3.1200	0.0099
96	2.603	0.0036	2.7900	0.0041	2.8800	0.0032	2.8150	0.0037	2.8723	0.0012	3.0200	0.0024	2.9800	0.0076

Table 1.11 *Effect of temperature Vs time intervals on the solubility profile of steroids in S-F formulations.*

	Hydrocortisone (S_i % w/v)				*Hydrocortisone acetate (S_i %w/v)*				*Testosterone acetate (S_i % w/v)*			
Time (hour)	25C°	SD	Pre-heated at 50°C	SD	25C°	SD	Pre-heated at 50°C	SD	25C°	SD	Pre-heated at 50°C	SD
0	0.926	0.001	1.691	0.006	0.135	0.008	0.160	0.005	1.146	0.003	3.904	0.002
6	0.880	0.003	1.470	0.006	0.168	0.008	0.162	0.006	1.480	0.004	3.245	0.002
12	0.888	0.002	1.453	0.006	0.170	0.008	0.160	0.006	1.567	0.004	2.910	0.003
24	0.860	0.011	1.412	0.006	0.164	0.007	0.159	0.003	1.717	0.004	2.925	0.002
36	0.862	0.011	1.334	0.007	0.160	0.008	0.157	0.002	1.917	0.005	2.850	0.003
48	0.891	0.010	1.251	0.007	0.154	0.008	0.144	0.005	2.034	0.004	2.645	0.006
96	0.896	0.001	1.215	0.006	0.146	0.007	0.143	0.004	2.075	0.003	2.399	0.005
192	0.901	0.009	1.097	0.006	0.145	0.007	0.141	0.003	2.179	0.002	2.301	0.006
384	0.903	0.008	1.098	0.007	0.145	0.007	0.139	0.005	2.377	0.003	2.299	0.004
576	0.915	0.008	1.077	0.007	0.144	0.007	0.136	0.003	2.390	0.003	2.218	0.004
768	0.926	0.009	1.048	0.006	0.142	0.002	0.137	0.005	2.410	0.001	2.240	0.004

	Testosterone (S_i % w/v)				*Progesterone (S_i% w/v)*			
Time (hrs)	25C°	SD	Pre-heated at 50°C	SD	25C°	SD	Pre-heated at 50°C	SD
0	1.046	0.005	3.873	0.007	1.825	0.005	3.261	0.004
6	2.240	0.003	3.336	0.005	2.089	0.004	2.907	0.006
12	2.349	0.004	3.360	0.003	2.11	0.002	2.694	0.004
24	2.450	0.006	3.220	0.003	2.345	0.004	2.583	0.004
36	2.56	0.004	3.090	0.008	2.648	0.003	2.467	0.003
48	2.591	0.005	2.917	0.005	2.681	0.008	2.375	0.004
96	2.603	0.004	2.815	0.004	2.744	0.005	2.298	0.006
192	2.610	0.003	2.70	0.002	2.727	0.002	2.230	0.006
384	2.651	0.004	2.635	0.004	2.710	0.008	2.235	0.008
576	2.721	0.005	2.601	0.002	2.715	0.004	2.211	0.005
768	2.742	0.006	2.591	0.004	2.710	0.005	2.143	0.008

Table 1.12 *Solubility (%w/v) of hydroxy benzoate derivatives in lipid excipients and/or SEDDS formulations at 25°C.*

	Methyl paraben (S_i % w/v)			Propyl paraben (S_i% w/v)			Butyl paraben (S_i % w/v)			Ethyl paraben (S_i % w/v)		
	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)	Mean (n=3)	SD (n=3)	RSD %(n=3)
Miglyol 812®	10.319	0.260	2.519	11.920	3.990	33.473	7.900	0.103	1.306	9.430	0.459	4.867
Imwitor 988®	26.870	0.960	3.573	15.740	4.070	25.858	9.320	0.667	7.157	24.160	1.670	6.912
Propylene glycol	31.840	0.545	1.712	12.480	0.622	4.984	11.990	0.370	3.085	29.570	4.077	13.788
*Surfactant-free	35.998	0.415	1.153	18.709	1.410	7.536	14.620	1.067	7.298	31.970	0.831	2.599
**Type II	25.270	1.800	7.123	14.335	0.838	5.846	9.270	0.388	4.186	20.920	2.030	9.704
***TypeIII A	19.419	0.506	2.606	19.198	0.306	1.595	9.990	0.429	4.294	24.938	1.936	7.763
****Type IIIB	50.976	1.450	2.844	21.152	0.376	1.778	12.610	2.230	17.684	30.557	2.230	7.298

*Type II Miglyol 812®+Tagat To®
40%:60%

**Surfactant-free Miglyol 812®+Imwitor 988®+PEG400
30%+50%+20%

***Type IIIA CRH 40®+Imwitor 988®+Miglyol 812®
35% + 35% + 30%

****Type IIIB CHR 40®+ Imwitor 988® + Miglyol 812® + PEG400
(1:9)
30%+30%+40%

Appendix 2

	Hydrocortisone				Hydrocortisone acetate				Testosterone			
Weight fraction of cosolvent	%w/v So of PEG 400	SD	%w/v So of PG	SD	%w/v So of PEG 400	SD	%w/v So of PG	SD	%w/v So of PEG 400	SD	%w/v So of PG	SD
0.1	0.0070	0.025	0.0088	0.0087	0.0007	0.0053	0.0006	0.0078	0.0013	0.0073	0.0010	0.0100
0.3	0.0071	0.025	0.0091	0.0087	0.0067	0.0135	0.0025	0.0130	0.0048	0.0043	0.0098	0.0125
0.5	0.0073	0.007	0.0092	0.0093	0.0067	0.0158	0.0026	0.0173	0.0103	0.0035	0.0099	0.0100
0.7	0.0075	0.008	0.0099	0.0097	0.0068	0.0058	0.0029	0.0180	0.0153	0.0028	0.0099	0.0125
0.9	0.0086	0.008	0.0101	0.0098	0.0077	0.0195	0.0039	0.0148	0.0215	0.0103	0.0106	0.0125
1.0	0.0113	0.008	0.0103	0.0098	0.0086	0.0203	0.0045	0.0183	0.0328	0.0205	0.0113	0.0125
1.5	0.0168	0.010	0.0171	0.0100	0.0134	0.0098	0.0049	0.0220	0.0410	0.0138	0.0212	0.0100
2.0	0.0232	0.010	0.0235	0.0103	0.0183	0.0163	0.0064	0.0225	0.0473	0.0070	0.0312	0.0100
2.5	0.0319	0.011	0.0353	0.0106	0.0233	0.0175	0.0076	0.0223	0.0533	0.0080	0.0738	0.0100
3.0	0.0382	0.011	0.0486	0.0113	0.0410	0.0133	0.0086	0.0245	0.0610	0.0048	0.0913	0.0125
3.5	0.0507	0.012	0.0612	0.0123	0.0510	0.0165	0.0095	0.0030	0.0663	0.0200	0.1286	0.0100
4.0	0.0650	0.003	0.0737	0.0128	0.0601	0.0030	0.0103	0.0108	0.0728	0.0128	0.1598	0.0125

	Testosterone acetate				Progesterone			
Weight fraction of cosolvent	%w/v So of PEG 400	SD	%w/v So of PG	SD	%w/v So of PEG 400	SD	%w/v So of PG	SD
0.1	0.0053	0.0033	0.0054	0.0183	0.0011	0.0031	0.0014	0.0056
0.3	0.0270	0.0026	0.0133	0.0230	0.0071	0.0039	0.0057	0.0034
0.5	0.0570	0.0030	0.0413	0.0138	0.0091	0.0060	0.0058	0.0024
0.7	0.0825	0.0034	0.0713	0.0173	0.0116	0.0138	0.0058	0.0026
0.9	0.0950	0.0048	0.0917	0.0220	0.0133	0.0031	0.0062	0.0024
1.0	0.1075	0.0034	0.1090	0.0288	0.0141	0.0089	0.0063	0.0099
1.5	0.1350	0.0030	0.1324	0.0113	0.0208	0.0036	0.0145	0.0105
2.0	0.1633	0.0044	0.1548	0.0238	0.0332	0.0026	0.0183	0.0046
2.5	0.1850	0.0030	0.1870	0.0230	0.0458	0.0041	0.0330	0.0036
3.0	0.2198	0.0034	0.2179	0.0288	0.0608	0.0056	0.0750	0.0029
3.5	0.2425	0.0038	0.2350	0.0230	0.0709	0.0135	0.1120	0.0053
4.0	0.2684	0.0027	0.3274	0.0173	0.0870	0.0031	0.1478	0.0083

Table 2.1 The solubility (% w/v) of steroids in aqueous solution of hydrophilic co-solvent (PG and/or PEG 400).

	Hydrocortisone				Hydrocortisone acetate				Testosterone			
Weight fraction of cosolvent	%w/v So of PEG 400	SD	%w/v So of PG	SD	%w/v So of PEG 400	SD	%w/v So of PG	SD	%w/v So of PEG 400	SD	%w/v So of PG	SD
0.1	0.0077	0.0153	0.0089	0.0085	0.00077	0.0113	0.0008	0.0028	0.0015	0.0026	0.0013	0.0103
0.3	0.0096	0.0158	0.0095	0.0135	0.0051	0.0165	0.0023	0.0128	0.0092	0.0032	0.0175	0.0031
0.5	0.0098	0.0135	0.0098	0.0110	0.0065	0.0195	0.0025	0.0168	0.0110	0.0043	0.0350	0.0125
0.7	0.0101	0.0140	0.0133	0.0135	0.0081	0.0035	0.0028	0.0230	0.0225	0.0041	0.0525	0.0083
0.9	0.0106	0.0158	0.0148	0.0080	0.0091	0.0160	0.0031	0.0195	0.0375	0.0039	0.0565	0.0036
1.0	0.0109	0.0143	0.0153	0.0088	0.0108	0.0058	0.0036	0.0195	0.0540	0.0053	0.0715	0.0117
1.5	0.0185	0.0128	0.0240	0.0053	0.0152	0.0123	0.0055	0.0113	0.0762	0.0056	0.0910	0.0138
2.0	0.0246	0.0103	0.0423	0.0178	0.0233	0.0145	0.0069	0.0188	0.0973	0.0079	0.1035	0.0057
2.5	0.0363	0.0163	0.0505	0.0055	0.0346	0.0208	0.0080	0.0183	0.1250	0.0067	0.1250	0.0084
3.0	0.0487	0.0178	0.0685	0.0080	0.0461	0.0138	0.0091	0.0203	0.1672	0.0080	0.1469	0.0026
3.5	0.0637	0.0143	0.0800	0.0168	0.0671	0.0183	0.0101	0.0230	0.2330	0.0103	0.1791	0.0028
4.0	0.0787	0.0155	0.0958	0.0118	0.0787	0.0070	0.0116	0.0133	0.3555	0.0089	0.1932	0.0058

	Testosterone acetate				Progesterone			
Weight fraction of cosolvent	%w/v So of PEG 400	SD	%w/v So of PG	SD	%w/v So of PEG 400	SD	%w/v So of PG	SD
0.1	0.0050	0.0555	0.0052	0.0828	0.0010	0.0034	0.0016	0.0335
0.3	0.0390	0.0200	0.0370	0.0280	0.00950	0.0077	0.0115	0.0765
0.5	0.0635	0.0200	0.0360	0.0570	0.0245	0.0055	0.0116	0.0548
0.7	0.1030	0.0225	0.0373	0.0343	0.0450	0.0103	0.0119	0.1028
0.9	0.0985	0.0300	0.0725	0.1055	0.0730	0.0128	0.0125	0.1280
1.0	0.1280	0.0400	0.0950	0.1280	0.0940	0.0028	0.0225	0.0278
1.5	0.1463	0.0573	0.1483	0.1505	0.1024	0.0041	0.0593	0.0413
2.0	0.1795	0.0685	0.1973	0.0520	0.1235	0.0048	0.1165	0.0483
2.5	0.1900	0.1530	0.2140	0.0258	0.1490	0.0071	0.1790	0.0710
3.0	0.2425	0.1255	0.2800	0.0910	0.1813	0.0083	0.2480	0.0833
3.5	0.3733	0.1158	0.4175	0.1778	0.2390	0.0026	0.3819	0.0263
4.0	0.4925	0.0263	0.5300	0.1303	0.3515	0.0074	0.6908	0.0738

Table 2.2 The solubility (% w/v) of steroids in tris-maleate buffer (pH 6.5)of hydrophilic co-solvent (PG and/or PEG 400).

	Hydrocortisone				Hydrocortisone acetate				Testosterone			
Weight fraction of surfactant	%w/v So of CRH40	SD	%w/v So of Tween 80	SD	%w/v So of CRH40	SD	%w/v So of Tween 80	SD	%w/v So of CRH40	SD	%w/v So of Tween 80	SD
0.1	0.0050	0.0050	0.0228	0.0017	0.0209	0.0150	0.0104	0.0110	0.0225	0.0100	0.0223	0.0083
0.3	0.0950	0.0106	0.0300	0.0095	0.0218	0.0075	0.0143	0.0150	0.0475	0.0225	0.0305	0.0218
0.5	0.1075	0.0107	0.0577	0.0014	0.0245	0.0225	0.0236	0.0073	0.1025	0.0103	0.1218	0.0033
0.7	0.1358	0.0110	0.0753	0.0200	0.0688	0.0050	0.0258	0.0040	0.1525	0.0035	0.1635	0.0233
0.9	0.1625	0.0110	0.1046	0.0525	0.1108	0.0050	0.0373	0.0028	0.2150	0.0178	0.1808	0.0113
1.0	0.1871	0.0112	0.1320	0.0158	0.1282	0.0175	0.0393	0.0093	0.3275	0.0100	0.2213	0.0205
1.5	0.2054	0.0113	0.1566	0.0178	0.1725	0.0175	0.0595	0.0213	0.4100	0.0225	0.2428	0.0173
2.0	0.2321	0.0115	0.1825	0.0108	0.1973	0.0150	0.1065	0.0248	0.4725	0.0030	0.2600	0.0028
2.5	0.2685	0.0116	0.2055	0.0145	0.2310	0.0225	0.1580	0.0153	0.5325	0.0128	0.4025	0.0095
3.0	0.3233	0.0118	0.2280	0.0625	0.2757	0.0225	0.1943	0.0178	0.6100	0.0030	0.4625	0.0525
3.5	0.3626	0.0119	0.2631	0.0228	0.3600	0.0150	0.2335	0.0158	0.6625	0.0143	0.5800	0.1550
4.0	0.4335	0.0120	0.3958	0.0185	0.4575	0.0125	0.2778	0.0183	0.7275	0.0203	0.7200	0.0450

	Testosterone acetate				Progesterone			
Weight fraction of surfactant	%w/v So of CRH40	SD	%w/v So of Tween 80	SD	%w/v So of CRH40	SD	%w/v So of Tween 80	SD
0.1	0.0228	0.0023	0.0275	0.0058	0.0153	0.0030	0.0180	0.0030
0.3	0.0533	0.0030	0.0748	0.0083	0.0248	0.0023	0.0300	0.0085
0.5	0.1080	0.0078	0.1125	0.0030	0.0358	0.0030	0.0523	0.0210
0.7	0.1530	0.0105	0.1608	0.0210	0.0640	0.0003	0.0733	0.0203
0.9	0.2113	0.0128	0.2308	0.0068	0.0998	0.0021	0.0975	0.0220
1.0	0.3000	0.0105	0.3075	0.0095	0.1503	0.0137	0.1283	0.0223
1.5	0.3625	0.0213	0.3900	0.0113	0.1998	0.0073	0.1500	0.0198
2.0	0.4200	0.0059	0.4725	0.0090	0.2475	0.0187	0.2005	0.0203
2.5	0.4800	0.0103	0.5250	0.0045	0.3275	0.0145	0.2335	0.0203
3.0	0.5325	0.0248	0.6025	0.0035	0.4125	0.0059	0.2725	0.0120
3.5	0.5750	0.0050	0.6675	0.0040	0.4725	0.0015	0.4330	0.0225
4.0	0.6275	0.0058	0.7450	0.0045	0.5600	0.0019	0.5200	0.0048

Table 2.3 The solubility (%w/v) of steroids in aqueous solution of hydrophilic surfactant (Tween 80® or/ and CRH 40®) at 25°C.

	Hydrocortisone				Hydrocortisone acetate				Testosterone			
Weight fraction of surfactant	%w/v So of CRH40	SD	%w/v So of Tween 80	SD	%w/v So of CRH40	SD	%w/v So of Tween 80	SD	%w/v So of CRH40	SD	%w/v So of Tween 80	SD
0.1	0.0173	0.0017	0.0190	0.0023	0.0613	0.0150	0.0803	0.0175	0.1030	0.0080	0.0240	0.0225
0.3	0.0600	0.0095	0.0325	0.0080	0.0855	0.0200	0.1143	0.0100	0.1788	0.0133	0.0330	0.0085
0.5	0.0860	0.0014	0.0718	0.0025	0.1063	0.0025	0.1498	0.0175	0.2275	0.0058	0.0885	0.0030
0.7	0.1220	0.0200	0.0878	0.0023	0.1499	0.0010	0.1608	0.0225	0.2486	0.0028	0.1948	0.0128
0.9	0.1464	0.0525	0.1225	0.0033	0.1780	0.0003	0.1973	0.0225	0.3223	0.0175	0.2488	0.0090
1.0	0.1695	0.0158	0.1400	0.0073	0.2000	0.0005	0.2110	0.0175	0.3425	0.0300	0.3781	0.0178
1.5	0.2550	0.0178	0.1600	0.0125	0.2478	0.0013	0.2488	0.0025	0.3775	0.1350	0.5825	0.0005
2.0	0.3412	0.0108	0.1780	0.0198	0.2973	0.0018	0.3235	0.0050	0.4850	0.1575	0.7050	0.0045
2.5	0.4035	0.0145	0.2085	0.0023	0.3535	0.0018	0.3998	0.0125	0.5525	0.0975	0.7825	0.0180
3.0	0.4890	0.0625	0.2303	0.0180	0.3975	0.0008	0.4550	0.0125	0.6750	0.1250	0.8550	0.0045
3.5	0.5100	0.0228	0.3000	0.0163	0.4450	0.0015	0.5348	0.0225	0.8000	0.0450	0.9275	0.0003
4.0	0.5575	0.0185	0.4300	0.0265	0.4813	0.0005	0.6450	0.0150	0.8750	0.1200	1.0225	0.0030

	Testosterone acetate				Progesterone			
Weight fraction of surfactant	%w/v So of CRH40	SD	%w/v So of Tween 80	SD	%w/v So of CRH40	SD	%w/v So of Tween 80	SD
0.1	0.0275	0.0006	0.0325	0.0075	0.0178	0.0123	0.0208	0.0135
0.3	0.0575	0.0053	0.0725	0.0105	0.0358	0.0160	0.0380	0.0133
0.5	0.1223	0.0068	0.1078	0.0130	0.0495	0.0212	0.0695	0.0158
0.7	0.1530	0.0060	0.1780	0.0178	0.0985	0.0122	0.0808	0.0130
0.9	0.2225	0.0055	0.2328	0.0128	0.1408	0.0217	0.1530	0.0030
1.0	0.3075	0.0048	0.3375	0.0030	0.2085	0.0217	0.1778	0.0055
1.5	0.3800	0.0078	0.4500	0.0105	0.2750	0.0125	0.2418	0.0140
2.0	0.4950	0.0040	0.6025	0.0153	0.3575	0.0125	0.3075	0.0030
2.5	0.5850	0.0070	0.6825	0.0085	0.4358	0.0160	0.3825	0.0218
3.0	0.6525	0.0085	0.7475	0.0183	0.4825	0.0113	0.4725	0.0208
3.5	0.7450	0.0195	0.8100	0.0168	0.5800	0.0110	0.5625	0.0133
4.0	0.8300	0.0030	0.8950	0.0085	0.6625	0.0150	0.6950	0.0228

Table 2.4 The solubility (%w/v) of steroids in tris-maleate buffer (pH 6.5) of hydrophilic surfactant (Tween 80® and/or CRH 40®) at 25°C.

	Hydrocortisone				Hydrocortisone acetate				Testosterone			
Weight fraction of formulation	%w/v So of M 812	SD	%w/v So of Type II	SD	%w/v So of M 812	SD	%w/v So of Type II	SD	%w/v So of M 812	SD	%w/v So of Type II	SD
0.1	0.00090	0.00003	0.00100	0.00001	0.00400	0.00775	0.00044	0.02200	0.00900	0.00900	0.00800	0.00230
0.3	0.00310	0.00013	0.00350	0.00010	0.01000	0.01300	0.00117	0.01900	0.02300	0.00720	0.01900	0.00173
0.5	0.00520	0.00004	0.00710	0.00011	0.02200	0.01725	0.00202	0.01350	0.04179	0.00720	0.03300	0.00345
0.7	0.00810	0.00005	0.00930	0.00014	0.02900	0.01800	0.00253	0.01900	0.05650	0.00900	0.05100	0.00288
0.9	0.00970	0.00006	0.01360	0.00016	0.03650	0.01475	0.00370	0.00925	0.06825	0.00720	0.06300	0.00173
1.0	0.01000	0.00006	0.01530	0.00019	0.03725	0.01825	0.00390	0.01625	0.07125	0.00900	0.06500	0.00230
1.5	0.01380	0.00007	0.01640	0.00021	0.05200	0.02200	0.00510	0.01650	0.09225	0.00900	0.09100	0.00173
2.0	0.01760	0.00008	0.01890	0.00023	0.08393	0.02250	0.00690	0.01275	0.13800	0.00900	0.13900	0.00173
2.5	0.01910	0.00008	0.02200	0.00027	0.10300	0.02225	0.00820	0.02275	0.19900	0.00900	0.18900	0.00127
3.0	0.02190	0.00008	0.02430	0.00032	0.13920	0.02450	0.01010	0.01475	0.27900	0.00720	0.22100	0.00230
3.5	0.02900	0.00009	0.02630	0.00036	0.18600	0.00300	0.01230	0.00250	0.31300	0.00720	0.26300	0.00288
4.0	0.03710	0.00010	0.02890	0.00043	0.22100	0.01075	0.01410	0.00850	0.34900	0.00900	0.30100	0.00220

	Testosterone acetate				Progesterone			
Weight fraction of formulation	%w/v So of M 812	SD	%w/v So of Type II	SD	%w/v So of M 812	SD	%w/v So of Type II	SD
0.1	0.0120	0.0050	0.0070	0.0058	0.0190	0.0035	0.0060	0.0083
0.3	0.0350	0.0080	0.0180	0.0105	0.0430	0.0120	0.0170	0.0133
0.5	0.0599	0.0053	0.0293	0.0030	0.0748	0.0105	0.0250	0.0095
0.7	0.0810	0.0128	0.0430	0.0103	0.0948	0.0010	0.0390	0.0105
0.9	0.1010	0.0053	0.0601	0.0155	0.1350	0.0145	0.0540	0.0038
1.0	0.1121	0.0055	0.0623	0.0030	0.1500	0.0009	0.0585	0.0045
1.5	0.1425	0.0003	0.0970	0.0028	0.1995	0.0122	0.0910	0.0028
2.0	0.2010	0.0230	0.1410	0.0035	0.3280	0.0113	0.1122	0.0093
2.5	0.2810	0.0035	0.1930	0.0130	0.3725	0.0223	0.1585	0.0058
3.0	0.3514	0.0045	0.2350	0.0073	0.4250	0.1225	0.1783	0.0065
3.5	0.4010	0.0058	0.2790	0.0128	0.5575	0.0203	0.2165	0.0045
4.0	0.4760	0.0105	0.3300	0.0155	0.6910	0.0113	0.2460	0.0060

Table 2.5 The solubility (w/v) of steroids in aqueous solution of type I (Miglyol 812®) and type II (Miglyol 812® and Tagat TO®)at 25°C.

	Hydrocortisone				Hydrocortisone acetate				Testosterone			
Weight fraction of formulation	%w/v So of M 812	SD	%w/v So of Type II	SD	%w/v So of M 812	SD	%w/v So of Type II	SD	%w/v So of M 812	SD	%w/v So of Type II	SD
0.1	0.00785	0.00022	0.00090	0.1375	0.0038	0.0155	0.000500	0.0185	0.0085	0.0008	0.0079	0.000079
0.3	0.00210	0.00019	0.00320	0.1525	0.0090	0.0135	0.001275	0.0165	0.0210	0.0003	0.0170	0.000170
0.5	0.00450	0.00023	0.00630	0.0800	0.0130	0.0180	0.001675	0.0048	0.0387	0.0004	0.0291	0.000291
0.7	0.00730	0.00011	0.00860	0.1775	0.0190	0.0160	0.001950	0.0050	0.0501	0.0002	0.0410	0.000410
0.9	0.00870	0.00016	0.01340	0.1275	0.0251	0.0195	0.002225	0.0128	0.0623	0.0003	0.0520	0.000520
1.0	0.00910	0.00020	0.01420	0.2075	0.0261	0.0223	0.002950	0.0133	0.0641	0.0006	0.0541	0.000541
1.5	0.01090	0.00012	0.01610	0.1300	0.0340	0.0053	0.003550	0.0150	0.0890	0.0002	0.0820	0.000820
2.0	0.01410	0.00017	0.01810	0.1525	0.0470	0.0025	0.004825	0.0068	0.1100	0.0004	0.1100	0.001100
2.5	0.01870	0.00017	0.02010	0.1900	0.0580	0.0140	0.006438	0.0153	0.1510	0.0002	0.1530	0.001530
3.0	0.02120	0.00013	0.02310	0.1525	0.0710	0.0198	0.008147	0.0113	0.1990	0.0002	0.1810	0.001810
3.5	0.02510	0.00015	0.02710	0.0575	0.0870	0.0203	0.009800	0.0220	0.2310	0.0003	0.2310	0.002310
4.0	0.02710	0.00018	0.03100	0.1600	0.1020	0.0030	0.012550	0.0230	0.2860	0.0002	0.2830	0.002830

	Testosterone acetate				Progesterone			
Weight fraction of formulation	%w/v So of M 812	SD	%w/v So of Type II	SD	%w/v So of M 812	SD	%w/v So of Type II	SD
0.1	0.0110	0.0030	0.0065	0.0153	0.0170	0.0028	0.0057	0.0143
0.3	0.0310	0.0080	0.0161	0.0205	0.0320	0.0068	0.0150	0.0030
0.5	0.0570	0.0885	0.0250	0.0058	0.0610	0.0105	0.0230	0.0011
0.7	0.0740	0.0100	0.0410	0.0103	0.0870	0.0070	0.0320	0.0055
0.9	0.0910	0.0163	0.0580	0.0045	0.1100	0.0043	0.0490	0.1025
1.0	0.0931	0.0080	0.0600	0.0038	0.1200	0.0030	0.0510	0.0098
1.5	0.1290	0.0113	0.0890	0.0040	0.1800	0.0053	0.0830	0.0103
2.0	0.1890	0.0168	0.1210	0.0205	0.2200	0.0108	0.1010	0.0145
2.5	0.2310	0.0085	0.1890	0.0030	0.2780	0.0133	0.1400	0.0045
3.0	0.2910	0.0130	0.2310	0.0038	0.3010	0.0200	0.1710	0.0038
3.5	0.3410	0.0133	0.2760	0.0028	0.3530	0.0168	0.2110	0.0080
4.0	0.3810	0.0160	0.3010	0.0048	0.3990	0.0178	0.2420	0.0128

Table 2.6 The solubility (%w/v) of steroids in tris-maleate buffer (pH 6.5) of type I (Miglyol 812®) and type II (Miglyol 812® and Tagat TO®) at 25°C.

	Hydrocortisone						Hydrocortisone acetate					
Weight fraction of formulation	%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD	%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD
0.1	0.0090	0.0108	0.0080	0.0058	0.0120	0.000120	0.00190	0.0128	0.0011	0.0001	0.00150	0.0375
0.3	0.0190	0.0163	0.0120	0.0059	0.0371	0.000371	0.00540	0.0158	0.0033	0.0002	0.00580	0.0450
0.5	0.0310	0.0108	0.0230	0.0059	0.0750	0.000750	0.00890	0.0030	0.0050	0.0000	0.00910	0.1575
0.7	0.0046	0.0183	0.0390	0.0059	0.0930	0.000930	0.01200	0.0055	0.0086	0.0024	0.01200	0.1725
0.9	0.0630	0.0203	0.0500	0.0059	0.1239	0.001239	0.01970	0.0245	0.0141	0.0001	0.01610	0.2250
1.0	0.0647	0.0161	0.0510	0.0059	0.1250	0.001250	0.02100	0.0128	0.0150	0.0005	0.01640	0.0325
1.5	0.0830	0.0193	0.0720	0.0061	0.1741	0.001741	0.02730	0.0058	0.0190	0.0003	0.02100	0.2075
2.0	0.0920	0.0031	0.0910	0.0063	0.0210	0.000210	0.03010	0.0190	0.0252	0.0023	0.03000	0.1550
2.5	0.1100	0.0119	0.1100	0.0064	0.0281	0.000281	0.03520	0.0048	0.0291	0.0017	0.03620	0.1425
3.0	0.1620	0.0168	0.1490	0.0066	0.0350	0.000350	0.03910	0.0120	0.0351	0.0012	0.04410	0.1550
3.5	0.2100	0.0032	0.1960	0.0068	0.0411	0.000411	0.04420	0.0083	0.0381	0.0014	0.05340	0.1025
4.0	0.2680	0.0158	0.2100	0.0070	0.4900	0.004900	0.05200	0.0148	0.0430	0.0003	0.06510	0.0375
	Testosterone						Testosterone acetate					
Weight fraction of formulation	%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD	%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD
0.1	0.0092	0.0015	0.0050	0.000050	0.0300	0.0125	0.0090	0.0030	0.0040	0.0030	0.0220	0.0025
0.3	0.0210	0.0050	0.0130	0.000130	0.0910	0.0300	0.0230	0.0550	0.0090	0.0058	0.0620	0.0075
0.5	0.0430	0.0025	0.0210	0.000210	0.1320	0.1125	0.0410	0.0028	0.0150	0.0105	0.1010	0.0100
0.7	0.0710	0.0058	0.0340	0.000340	0.1900	0.0030	0.0610	0.0038	0.0260	0.0031	0.1530	0.0050
0.9	0.0967	0.0175	0.0510	0.000510	0.2400	0.0058	0.0967	0.0030	0.0380	0.0131	0.2120	0.0223
1.0	0.0980	0.0025	0.0520	0.000520	0.2500	0.0085	0.0980	0.0035	0.0410	0.1050	0.2140	0.0058
1.5	0.1310	0.0075	0.0690	0.000690	0.4100	0.0108	0.1200	0.0045	0.0600	0.1050	0.3200	0.0113
2.0	0.1770	0.0125	0.0810	0.000810	0.5800	0.0135	0.1720	0.0030	0.0750	0.0141	0.4170	0.0070
2.5	0.2500	0.0075	0.0930	0.000930	0.7400	0.0085	0.2130	0.0035	0.0920	0.0185	0.5210	0.0040
3.0	0.2900	0.0150	0.0120	0.000120	0.9100	0.0025	0.2510	0.0048	0.1100	0.1550	0.6300	0.0035
3.5	0.3210	0.0110	0.0163	0.000163	1.0300	0.0058	0.2920	0.0023	0.1520	0.0035	0.7250	0.0040
4.0	0.3600	0.0025	0.0195	0.000195	1.2300	0.0080	0.3560	0.0033	0.1990	0.0128	0.8630	0.0030

Table 2.7 The solubility (%w/v) of steroids in aqueous solution of type IIIA (Miglyol 812®+ Imwitor 988® + CRH40®), type III B (Miglyol 812®+ Imwitor 988® + CRH40® + PG) and surfactant free formulation (Miglyol 812®+ Imwitor 988® + PG) at 25°C.

	Hydrocortisone						Hydrocortisone acetate					
Weight fraction of formulation	%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD	%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD
0.1	0.00870	0.0003	0.00770	0.00055	0.0110	0.0120	0.00170	0.0085	0.00110	0.000011	0.00149	0.000015
0.3	0.00172	0.0016	0.01300	0.00067	0.0330	0.0153	0.05210	0.0073	0.00325	0.000033	0.05200	0.000520
0.5	0.02900	0.0011	0.02200	0.00023	0.053	0.0208	0.00821	0.0245	0.00525	0.000053	0.00891	0.000089
0.7	0.04100	0.0020	0.03410	0.00076	0.0780	0.0215	0.00110	0.0135	0.09213	0.000921	0.01230	0.000123
0.9	0.05800	0.0020	0.04780	0.00093	0.0990	0.0128	0.01710	0.0080	0.01250	0.000125	0.01580	0.000158
1.0	0.05910	0.0021	0.04990	0.001	0.1090	0.0080	0.01930	0.0133	0.01380	0.000138	0.01600	0.000160
1.5	0.08800	0.0015	0.08600	0.00054	0.1600	0.0168	0.02430	0.0083	0.01690	0.000169	0.02210	0.000221
2.0	0.11200	0.0023	0.11000	0.0078	0.2210	0.0112	0.03010	0.0053	0.02090	0.000209	0.03110	0.000311
2.5	0.14900	0.0012	0.15000	0.0031	0.2800	0.0228	0.03910	0.0105	0.02510	0.000251	0.04230	0.000423
3.0	0.18700	0.0083	0.17200	0.0033	0.3340	0.0925	0.04810	0.0210	0.03410	0.000341	0.04780	0.000478
3.5	0.22100	0.0015	0.19900	0.0094	0.3910	0.0723	0.05770	0.0238	0.03890	0.000389	0.05120	0.000512
4.0	0.25900	0.0021	0.23100	0.0077	0.4560	0.0190	0.06340	0.0065	0.04490	0.000449	0.05640	0.000564
	Testosterone						Testosterone acetate					
Weight fraction of formulation	%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD	%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD
0.1	0.00870	0.000087	0.00560	0.0025	0.0360	0.000360	0.0080	0.0025	0.00330	0.0085	0.0230	0.0030
0.3	0.02100	0.000210	0.01430	0.0075	0.0980	0.000980	0.0200	0.0133	0.00610	0.0056	0.0640	0.0105
0.5	0.04700	0.000470	0.02710	0.0050	0.1410	0.001410	0.0452	0.0158	0.01100	0.0133	0.0912	0.0155
0.7	0.06900	0.000690	0.03510	0.0105	0.2210	0.002210	0.0684	0.0158	0.02310	0.0160	0.1420	0.0208
0.9	0.08200	0.000820	0.04760	0.0163	0.3210	0.003210	0.0850	0.0223	0.03000	0.0153	0.2010	0.0230
1.0	0.08750	0.000875	0.05210	0.0053	0.3580	0.003580	0.0920	0.0030	0.03250	0.0110	0.2360	0.0183
1.5	0.11000	0.001100	0.07710	0.0129	0.4200	0.004200	0.1120	0.0213	0.04540	0.0168	0.3510	0.0123
2.0	0.15200	0.001520	0.09310	0.0183	0.5610	0.005610	0.1580	0.0223	0.06200	0.0172	0.5120	0.0203
2.5	0.18600	0.001860	0.12100	0.0140	0.6860	0.006860	0.1930	0.0030	0.08900	0.0230	0.5780	0.0180
3.0	0.21100	0.002110	0.15500	0.0030	0.7810	0.007810	0.2210	0.0113	0.12100	0.0450	0.6690	0.0156
3.5	0.24900	0.002490	0.18900	0.0203	0.8300	0.008300	0.2650	0.0188	0.15300	0.0130	0.7910	0.0108
4.0	0.27900	0.002790	0.22700	0.0230	0.9190	0.009190	0.2990	0.0183	0.18700	0.0025	0.9210	0.0178

Table 2.8 The solubility (% w/v)of steroids in tris-maleate buffer (pH 6.5) of type IIIA (Miglyol 812®+ Imwitor 988® + CRH40®), type III B (Miglyol 812®+ Imwitor 988® + CRH40® + PG) and surfactant free formulation (Miglyol 812®+ Imwitor 988® + PG) at 25°C.

Progesterone (H_2O)						Progesterone (tris-maleate)					
%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD	%w/v So of Type III A	SD	%w/v So of Type III B	SD	%w/v So of S-F	SD
0.0110	0.0080	0.0065	0.0011	0.0210	0.0055	0.0130	0.0078	0.00650	0.0030	0.0230	0.0095
0.0467	0.0014	0.0120	0.0058	0.0630	0.0108	0.0331	0.0060	0.01020	0.0055	0.0674	0.0108
0.0690	0.0098	0.0270	0.0053	0.1010	0.0053	0.0612	0.0043	0.02830	0.0130	0.1100	0.0133
0.0843	0.0130	0.0510	0.0223	0.1530	0.0088	0.0930	0.0128	0.04140	0.0133	0.1720	0.0213
0.0998	0.0215	0.0750	0.0035	0.1920	0.0095	0.1120	0.0078	0.06210	0.0108	0.2030	0.0053
0.1020	0.0245	0.0770	0.0128	0.2210	0.0065	0.1340	0.0155	0.06600	0.0153	0.2410	0.0133
0.1620	0.0030	0.0930	0.0148	0.3130	0.0053	0.1930	0.0211	0.09210	0.0045	0.3490	0.0195
0.2100	0.0108	0.1230	0.0108	0.4000	0.0065	0.2610	0.0130	0.12700	0.0123	0.4820	0.0030
0.2730	0.0130	0.1780	0.0147	0.5120	0.0093	0.3160	0.0163	0.16900	0.0108	0.5620	0.0145
0.3310	0.0219	0.2220	0.0035	0.6200	0.0063	0.3520	0.0035	0.19500	0.0143	0.6730	0.0130
0.3860	0.0163	0.2580	0.0108	0.7230	0.0073	0.3800	0.0133	0.23700	0.0085	0.7990	0.0103
0.4250	0.0030	0.2890	0.0158	0.8470	0.0080	0.4310	0.0153	0.27890	0.0700	0.9170	0.0058

Table 2.9 The solubility (%w/v) of progesterone in aqueous solution or/ and tris-maleate buffer (pH 6.5) of type IIIA (Miglyo 812®+ Imwitor 988® + CRH40®), type III B (Miglyol 812®+ Imwitor 988® + CRH40® + PG) and surfactant free formulatio (Miglyol 812®+ Imwitor 988® + PG) at 25°C.

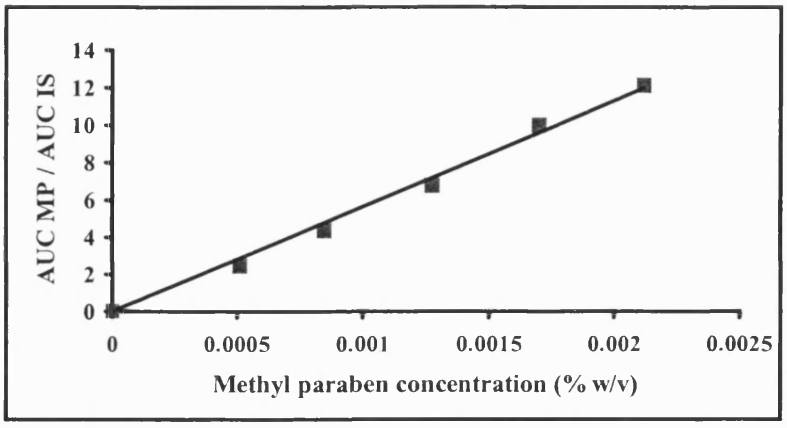
*SD stands for the standard deviation of three experiments repetitively.

Appendix 3

Methyl paraben

A series of methyl paraben solutions was prepared by dilution with acetonitrile 99%v/v. Samples were produced which contained (0.212, 0.5088, 0.848, 1.272, and 1.696) mg % of methyl paraben. Hydrocortisone was added as an internal standard in concentration 0.653 mg%. An analysis was performed as described in 6.2.2.4. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

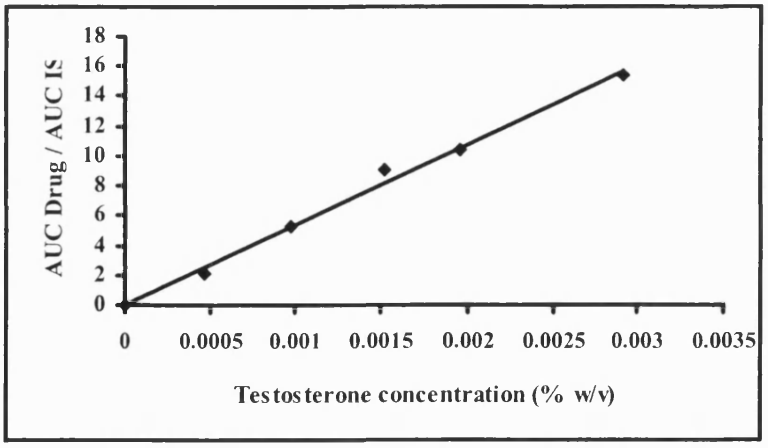
Slope = 5641.1X $\quad$ $\mathbf{r^2}$ = 0.9931 $\quad$ Intercept = 0



Testosterone

A series of testosterone solutions was prepared by dilution with methanol 99%v/v. Samples were produced which contained (0.468,0.976, 1.52, 1.952 and 2.92) mg % of methyl paraben. Dexamethasone was added as an internal standard in concentration 1.332 mg %. An analysis was performed as described in 6.2.2.4. A Beer--Lambert plot for concentration (% w/v) was determined with following regression analysis.

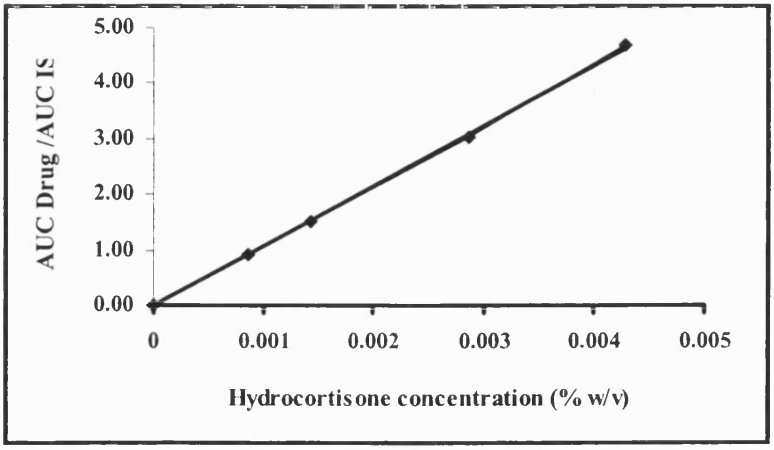
Slope = 5374.3X $\quad$ $\mathbf{r^2}$ = 0.9932 $\quad$ Intercept = 0



Hydrocortisone

A series of hydrocortisone solutions was prepared by dilution with methanol 99% v/v. Samples were produced which contained (0.122,0.917,1.528, 2.75,and 3.056) mg % hydrocortisone. Corticosteroids was added as an internal standard in concentration 0.682 mg %. An analysis was performed as described in 6.2.2.4. A Beer-Lambert plot for concentration (% w/v) was determined with following regression analysis.

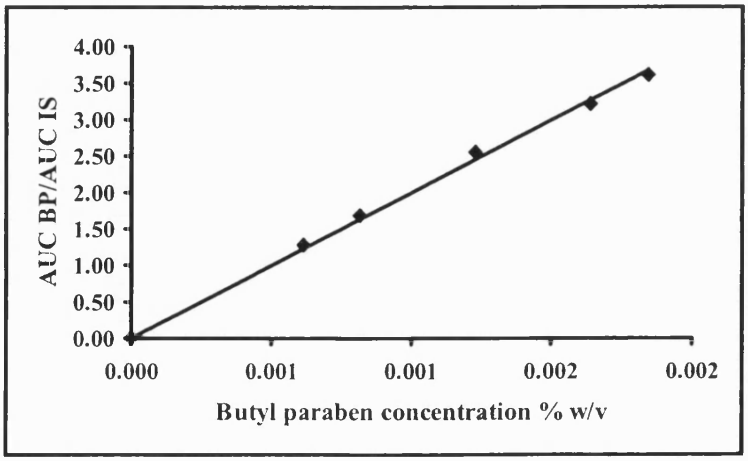
Slope = 1077.6X $\quad$ $\mathbf{r^2}$ = 0.9994 $\quad$ Intercept = 0



Butyl paraben

A series of butyl paraben solutions was prepared by dilution with acetonitrile 99%v/v. Samples were produced which contained (0.6312, 1.26, 1.89, 2.52 and 2.832) mg % of methyl paraben. Testosterone was added as an internal standard in concentration 5.364 mg %. An analysis was performed as described in 6.2.2.4. A Beer-Lambert plot for concentration (% w/v) was determined using regression analysis.

Slope = 1990X $\quad$ $\mathbf{r^2}$ = 0.9975 $\quad$ Intercept = 0



	Corn oil 5 min	CV	Corn oil 30 min	CV	Corn oil without calcium	CV	Miglyol 812 5min	CV	Miglyol 812 30min	CV
Oily phase	0.0895	0.514	0.079	1.44	0.060	2.217	0.104	0.646	0.093	2.311
Milky thick phase	0.0642	4.143	0.081	0.519	0.092	3.509				
Clear slightly turbid phase							0.098	0.935	0.096	2.554
Pellet phase							0.030	6.126	0.039	0.642
Mass recovered	0.154		0.160		0.152		0.232		0.228	
Mass balanced	0.156		0.171		0.173		0.237		0.238	
% recovered	98.47		93.72		88.04		98.01		95.73	
% digested	5.96		5.96		4.01		19.90		19.90	

	Miglyol oil without calcium	CV	Imwitor 988 5 min	CV	Imwitor 988 30 min	CV
Oily phase	0.125	1.869	0.054	6.010	0.047	2.340
Clear slightly turbid phase	0.106	1.383	0.015	9.670	0.016	5.100
Viscous , gel-like phase			0.147	0.133	0.151	3.110
Pellet phase	0.030	2.762	0.117	2.120	0.109	4.210
Mass recovered	0.261		0.333		0.324	
Mass balanced	0.212		0.322		0.322	
% recovered	122.65		103.60		100.50	
% digested	11.30		24.76		24.76	

Table 3.1 The amount (% w/v) of butyl paraben recovered of lipid excipients (LCT, MCT, mixed mono-, di-, and tri-glycerides) in standard pH-stat solution under standard pH-state conditions.

	Type II 5min	CV	Type II 30min	CV	Type IIIA 5min	CV	Type IIIA 30min	CV	Type III B 5min	CV	Type III B 30min	CV
oily phase	0.055	2.40	0.048	1.28								
Interface (slightly turbid phase)	0.029	2.26	0.038	0.99	0.079	0.97	0.084	2.74				
Subphase (micellar phase)	0.182	2.20	0.189	2.56	0.213	0.15	0.231	0.85	0.4520	4.40	0.4590	0.68
Pellet phase	0.046	2.07	0.049	3.48	0.091	1.85	0.101	1.58				
Mass recovered	0.312		0.324		0.382		0.416		0.452		0.459	
Mass balanced	0.325		0.378		0.443		0.466		0.463		0.460	
% recovered	96.02		85.65		86.37		89.43		97.60		99.75	
% digested	27.00		27.00		21.30		21.30		15.03		15.01	

	S-F 5min	CV	S-F 30 min	CV
Interface (slightly turbid phase)	0.112	1.07	0.123	3.00
Subphase (micellar phase)	0.248	0.43	0.240	1.84
Pellet phase	0.072	0.44	0.132	0.48
Mass recovered	0.432		0.495	
Mass balanced	0.453		0.507	
% recovered	95.22		97.63	
% digested	33.09		33.09	

Table 3.2 The amount (% w/v) of butyl paraben recovered of SEDDS formulations in standard pH-stat solution under standard pH-state conditions.

	I 988: M812 3:7 (5min)	CV	I 988: M812 3 :7 (30min)	CV	I 988: M812 7:3 (5min)	CV	I 988: M812 7:3 (30min)	CV	I 988: M812 5:5 (5min)	CV	I 988: M812 5:5 (30min)	CV
Interface (slightly turbid phase)	0.060	0.49	0.030	5.243	0.025	3.43	0.027	0.95	0.031	0.73	0.029	2.90
Subphase (micellar phase)	0.173	0.30	0.216	3.892	0.036	9.96	0.021	0.63				
viscous , gel-like phase					0.150	0.69	0.160	0.82	0.232	3.30	0.215	3.21
Pellet phase	0.060	1.29	0.067	1.495	0.058	1.82	0.065	0.18	0.067	1.70	0.091	1.31
Mass recovered	0.293		0.313		0.269		0.272		0.329		0.335	
Mass balanced	0.298		0.299		0.279		0.272		0.317		0.341	
% recovered	98.48		104.59		96.42		100.14		103.84		98.24	
% digested	20.65		20.65		33.75		33.75		27.63		27.63	

	I 988: M812 5:3 (5min)	CV	I 988: M812 5:3 (30min)	CV
Interface (slightly turbid phase)	0.031	0.12	0.034	1.20
Subphase (micellar phase)	0.217	1.31	0.229	1.70
Pellet phase	0.064	1.31	0.065	1.70
Mass recovered	0.248		0.263	
Mass balanced	0.278		0.281	
% recovered	89.16		93.76	
% digested	53.33		53.33	

Table 3.3 The amount (% w/v) of butyl paraben recovered of mixtures of MCT and mixed mono-, di-, and tri-glycerides in standard pH-stat solution under standard pH-state conditions.

	Corn oil 5 min	CV	Corn oil 30 min	CV	Corn oil without calcium	CV	Miglyol 812 5 min	CV	Miglyol 812 30 min	CV
Oily phase	0.0005	3.977	0.0008	0.875	0.0012	15.26	0.0009	0.548	0.0002	5.339
Milky turbid phase	0.0252	8.207	0.0261	20.627	0.0052	20.57				
Subphase (micellar phase)							0.0097	5.386	0.0087	11.602
Pellet phase							0.0213		0.0237	3.259
Mass recovered	0.0257		0.0269		0.0064		0.3190		0.0325	
Mass balanced	0.0292		0.0304		0.0068		0.3430		0.0343	
% recovered	88.185		88.622		95.12		93.00		94.856	
% digested	5.690		5.690		3.90		11.03		11.03	

	Imwitor 988 without calcium	CV	Imwitor 988 5 min	CV	Imwitor 988 30 min	CV
Oily phase	0.0002	5.339	0.0001	1.607	0.0001	1.607
Subphase (micellar phase)	0.0097	11.602	0.0075	3.353	0.0075	3.353
Viscous , gel-like phase	0.0193	3.259	0.0238	3.873	0.0238	3.873
Mass recovered	0.0292		0.0314		0.031	
Mass balanced	93.818		0.0301		0.031	
% recovered	0.0311		104.26		100.73	
% digested	12.070		25.19		25.19	

Table 3.4 The amount (% w/v) of hydrocortisone recovered of lipid excipients (LCT, MCT, mixed mono-, di-, and tri-glycerides) in standard pH-stat solution under standard pH-state conditions.

	Type II 5min	CV	Type II 30min	CV	Type IIIA 5min	CV	Type IIIA 30min	CV	Type III B 5min	CV	Type III B 30min	CV
Oily phase	0.00187	6.676	0.006	4.320								
Interface (slightly turbid phase)	0.01447	9.559	0.0301	1.37	0.0033	6.361	0.0046	0.139				
Subphase (micellar phase)	0.00957	2.510			0.0193	22.618	0.0153	1.101	0.0365	11.84	0.0372	9.2
Pellet phase												
Mass recovered	0.0259		0.0362		0.0225		0.0199		0.0365		0.0372	
Mass balanced	0.0355		0.0405		0.0191		0.0221		0.0361		0.0325	
% recovered	73.06		89.45		117.92		90.10		101.35		114.34	
% digested	8.24		8.24		29.78		29.78		21.23		21.23	

	S-F 5min	CV	S-F 30 min	CV
Interface (slightly turbid phase)	0.0043	2.000	0.0001	3.230
Subphase (micellar phase)	0.0151	1.933	0.0194	2.456
Pellet phase	0.0034	2.135	0.0054	1.228
Mass recovered	0.0227		0.0249	
Mass balanced	0.0239		0.0227	
% recovered	95.27		109.84	
% digested	25.43		25.43	

Table 3.5 The amount (% w/v) of hydrocortisone recovered of SEDDS formulations in standard pH-stat solution under standard pH-state conditions.

	I 988: M812 3:7 (5min)	CV	I 988: M812 3:7 (30min)	CV	I 988: M812 7:3 (5min)	CV	I 988: M812 7:3 (30min)	CV	I 988: M812 5:5 (5min)	CV	I 988: M812 5:5 (30min)	CV
Interface (slightly turbid phase)	0.0599	0.492	0.0295	17.324	0.0361	9.962	0.0211					
Subphase (micellar phase)	0.0183	0.297	0.0216	12.818	0.0252	3.430	0.0265	31.270	0.0307	15.88	0.0287	15.758
Viscous , gel-like phase					0.1504	0.685	0.1597	9.407	0.2320	15.78	0.2150	7.829
Pellet phase	0.0603	1.290	0.0672	13.450	0.0576	1.822	0.0649	10.417	0.0667	11.23	0.0912	2.530
Mass recovered	0.138		0.118		0.269		0.272		0.329		0.335	
Mass balanced	0.134		0.131		0.219		0.288		0.345		0.326	
% recovered	103.23		90.34		122.75		94.52		95.41		102.61	
% digested	14.93		14.93		39.28		39.28		26.47		26.47	

	I 988: M812 5:3 (5min)	CV	I 988: M812 5:3 (30min)	CV
Interface (slightly turbid phase)	0.0016	9.223	0.0021	2.427
Subphase (micellar phase)	0.0226	3.670	0.0231	14.248
Pellet phase	0.0044	1.420	0.0041	5.350
Mass recovered	0.0285		0.0293	
Mass balanced	0.029		0.028	
% recovered	100.13		100.13	
% digested	35.32		35.32	

Table 3.6 The amount (% w/v) of hydrocortisone recovered of mixtures of MCT and mixed mono-, di-, and tri-glycerides in standard pH-stat solution under standard pH-state conditions.

	Corn oil	CV	Miglyol 812	CV	Imwitor 988	CV
Oily phase	0.0425	1.245	0.066	43.151	0.003	1.925
Milky thick turbid phase	0.0808	0.915	0.012	6.414	0.076	22.278
Viscous , gel-like phase					0.362	5.756
Pellet phase			0.088	2.969		
Mass recovered	0.123		0.166		0.442	
Mass balanced	0.125		0.169		0.475	
% Recovered	98.94		98.09		93.13	
% Digested	7.69		13.75		23.74	

Table 3.7 The amount (% w/v) of methyl paraben recovered of mixtures of MCT and mixed mono-, di-, and tri-glycerides in standard pH-stat solution under standard pH-state conditions.

	S-F	CV	Type IIIA	CV	Type III B	CV	Type II	CV
Oily phase							0.032	3.069
Interface (slightly turbid phase)	0.124	4.077			0.534	3.362	0.292	4.434
Subphase (micellar phase)	0.396	1.421	0.382	5.527				
Pellet phase			0.042	4.014			0.083	4.359
Mass recovered	0.520		0.423		0.534		0.407	
Mass balanced	0.572		0.456		0.556		0.433	
% Recovered	90.92		92.74		96.04		93.97	
% Digested	19.29		29.69		9.32		29.13	

Table 3.9 The amount (% w/v) of methyl paraben recovered of SEDDS formulations in standard pH-stat solution under standard pH-state conditions.

	I 988: M812		I 988: M812		I 988: M812		I 988: M812	
	3 : 2	CV	3 : 7	CV	5 :5	CV	7 : 3	CV
Oily phase			0.073	9.102	0.025	1.117		
Interface (slightly turbid phase)	0.037	4.315	0.316	7.742	0.016	8.525	0.071	1.246
Subphase (micellar phase)	0.037	0.607						
Viscous, gel-like phase					0.377	0.391	0.302	2.638
Pellet phase			0.068	8.494			0.023	7.515
Mass recovered	0.075		0.384		0.393		0.396	
Mass balanced	0.090		0.401		0.377		0.386	
% Recovered	83.32		95.89		104.21		102.64	
% Digested	36.14		24.21		21.98		20.67	

Table 3.9 The amount (% w/v) of methyl paraben recovered of mixtures of MCT and mixed mono-, di-, and tri-glycerides in standard pH-stat solution under standard pH-state conditions .

	Corn oil		Miglyol 812		Imwitor 988	
		CV		CV		CV
Oily phase	0.004	2.145	0.006	12.656	0.00005	0.745
Milky thick turbid phase	0.0071	0.533	0.014	0.228		
Viscous , gel-like phase					0.002	6.628
Pellet phase			0.0003	2.300	0.030	2.652
Mass recovered	0.0114		0.0201		0.033	
Mass balanced	0.0123		0.0200		0.033	
% recovered	92.60		100.68		100.11	
% digested	7.04		14.39		22.65	

Table 3.10 The amount (% w/v) of testosterone recovered of mixtures of MCT and mixed mono-, di-, and tri-glycerides in standard pH-stat solution under standard pH-state conditions.

	S-F	CV	Type IIIA	CV	Type III B	CV	Type II	CV
Oily phase							0.0155	4.779
Interface (slightly turbid phase)	0.0147	1.253	0.0026	1.300				
Subphase (micellar phase)	0.0420	0.647	0.0493	1.312	0.0527	1.425	0.02961	14.858
Pellet phase	0.0018	1.366	0.0027	0.373				
Mass recovered	0.058		0.055		0.053		0.045	
Mass balanced	0.058		0.058		0.051		0.049	
% recovered	100.11		94.75		102.50		92.47	
% digested	39.54		30.17		8.93		25.67	

Table 3.11 The amount (% w/v) of testosterone recovered of SEDDS formulations in standard pH-stat solution under standard pH-state conditions.

	I 988: M812 3:2	CV	I 988: M812 7:3	CV	I 988: M812 5:5	CV	I 988: M812 3:7	CV
Interface (slightly turbid phase)	0.0289	2.140	0.0026	2.221	0.0021	11.857	0.0062	1.503
Subphase (micellar phase)	0.0365	6.360					0.0233	9.161
Viscous, gel-like phase			0.0401	18.740	0.0235	3.872		
Pellet phase			0.0075	10.718	0.0013	5.755	0.0016	10.970
Mass recovered	0.065		0.050		0.027		0.031	
Mass balanced	0.070		0.055		0.033		0.036	
% recovered	93.970		91.038		81.537		87.044	
% digested	31.130		24.640		26.040		17.980	

Table 3.12 The amount (% w/v) of testosterone recovered of mixtures of MCT and mixed mono-, di-, and tri-glycerides in standard pH-stat solution under standard pH-state conditions